THE REGULATION OF EXPRESSION OF TERMINAL DEOXYNUCLEOTIDYL TRANSFERASE IN AN INDUCIBLE MURINE PRE-B CELL LINE

By

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To My Family

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By

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Chairman: Edward J. Siden, Ph.D.
Major Department: Immunology and Medical Microbiology

Terminal deoxynucleotidyl transferase (Tdt) is a unique, lymphoid-specific enzyme capable of template independent deoxynucleotide polymerization. The function of the enzyme is unclear, although recent data strongly imply a role in the somatic diversification of immunoglobulin genes in pre-B lymphocytes.

The purpose of this study was to understand the control of synthesis of Tdt at a molecular level, utilizing the only class of murine B-lineage cell lines inducible for the enzyme. FLE1-4, an Abelson virus transformed fetal liver cell line, when treated with dibutyryl cAMP and caffeine (or other phosphodiesterase inhibitors), shows a three- to tenfold induction of Tdt, as measured by immunoprecipitation of pulse labelled enzyme, activity assays, and steady-state northern blot

analyses of mRNA levels. In addition, the study demonstrated the reversible nature of terminal transferase induction and allowed the calculation of a Tdt mRNA half-life in these cells of approximately 10.5 hours.

The study also revealed Tdt mRNA induction by a group of agents which inhibit DNA synthesis by various mechanisms. This finding implied that Tdt expression is unlinked to clonal proliferation of pre-B cells, and suggested a multistage model for B cell differentiation and the generation of immunoglobulin somatic variation. At a molecular level, these agents which induce Tdt were also shown to increase intracellular cAMP levels shortly before Tdt transcription. Finally, an analysis of histone phosphorylation patterns in induced cells revealed a marked enhancement of histone H1 phosphorylation, a finding which may be related to the induction event.

CHAPTER I

INTRODUCTION AND LITERATURE REVIEW

Every protein has a specific function. If we fail to understand that function the flaw is in human nature, not in Nature itself.

--F. J. Bollum (1982, p. 1)

Terminal Deoxynucleotidyl Transferase

During the late 1950s and early 1960s, when molecular biology was not yet considered an independent field of concentration, much study was undertaken in an effort to understand DNA replication. The DNA structural studies of Watson, Crick, and others had not only revealed a helical arrangement of complementary nucleotides but also suggested a mechanism of self-replication. The discovery of DNA polymerase I in E. coli by Kornberg, Bessman, and others in 1956 (Kornberg, 1980) prompted many investigators to seek analogous eukaryotic polymerases. The multiplicity and variety of DNA polymerases discovered in eukaryotic cells and tissues was not anticipated, especially because, until 1970, only one DNA polymerase had been identified in bacterial cells. After some charac-

terization of an inducible rat liver polymerase, F.J. Bollum chose to switch to calf thymus as a source of the enzyme, due either to the high nucleus to cytoplasm ratio, high rate of DNA synthesis, or, more likely, the readily available tissue supply--a necessity for large scale purification. Among the DNA polymerases isolated from the calf thymus was one unable to copy a template. Rather, it produced a DNA polymer for which the composition was determined by the concentration of the deoxynucleoside triphosphates present in the reaction mixture (Bollum, 1960, 1962). The enzyme activity was chromatographically separable from normal template-directed polymerization (Bollum et al., 1964), and a characteristic 3' terminal addition to an initiator polynucleotide was defined (Bollum, 1962). The new polymerase was named "terminal deoxynucleotidyl transferase."

Bollum's decision to use the thymus, of all possible tissues, was indeed fortuitous. A detailed survey of fetal bovine organs and tissues revealed that Tdt activity is restricted to the thymus. Furthermore, among major organs in the pig, rat, chicken, human, and rabbit systems, only the thymuses possessed demonstrable Tdt activity (Chang, 1971). In the same study, human bone marrow was described as lacking Tdt. More recent studies, using more sensitive assays, have demonstrated a

small, yet detectable population of Tdt^+ bone marrow cells. Thus far, Tdt has been demonstrated in all animals possessing a thymus or thymic rudiment.

Terminal transferase is present in the calf thymus at a concentration of 5-10 mg/kg of fresh tissue (Bollum, 1978). Purification to homogeneity originally revealed a peptide of native molecular weight 32,000 daltons (equilibrium centrifugation) comprised of two subunits of 26,500 and 8,000 daltons (Chang and Bollum, 1982). Antibody raised in rabbits against this homogeneous Tdt preparation detected a predominant 58,000 dalton protein in an enzyme preparation isolated in the presence of protease inhibitors (Silverstone et al., 1976). Moreover, a tryptic digestion of the 58 kD protein produced the two small immunoreactive subunits, as seen by western blot analysis (Nakamura et al., 1982). This group prepared calf, mouse, and rat thymic Tdt in the presence of protease inhibitors using immunoadsorbant chromatography, and based upon two-dimensional peptide analysis, concluded that the subunits previously reported were indeed the products of proteolysis, probably caused by degradation during the purification process. Wolf et al. (1982) demonstrated immunoprecipitation of a 58,000 dalton protein from *in vitro* translated calf thymus mRNA, the first report of cell free Tdt translation. Further,

the precipitation of the *in vitro* translation product could be competitively inhibited by the addition of purified calf Tdt. The high molecular weight species of Tdt (50,000-60,000 daltons) has been demonstrated in thymic tissue from nearly all mammals, chickens (Penit et al., 1982), amphibians (Brown, 1981) and in certain murine and human leukemic cells.

Biochemically, Tdt mediates deoxyribonucleotide polymerization in much the same manner as other eukaryotic polymerases. That is, 5′ nucleoside triphosphate addition occurs by polymerization onto a 3′-hydroxyl end of a DNA primer, and proceeds in a 5′--> 3′ direction. The notable difference with Tdt is the lack of template direction. Terminal transferase does require a single-stranded initiator of at least three deoxynucleoside monophosphate residues, with a free 3′-hydroxyl terminus. The K_m of the enzyme has previously been shown to decrease as the length of the initiator increases (from three up to seven or eight bases. The K_m is constant for initiators longer than eight nucleotides (Kato et al., 1967; Deibel and Coleman, 1980). There is a marked preference for single-stranded DNA initiators, as addition onto blunt-ended double-stranded DNA is very low (Michelson and Orkin, 1982). Kinetic analyses of Tdt activity have revealed a random sequential mechanism,

with the enzyme binding its two substrates in a random order (Deibel and Coleman, 1980). This group also reported that no significant differences exist in the biochemical kinetic characteristics betweeen calf thymus and human lymphoblastic Tdt, despite some structural variations (Deibel and Coleman, 1979).

A profound inhibition of Tdt activity by ATP differentiates this enzyme from all other reported DNA polymerases, including reverse transcriptase (Kato et al., 1967; Modak, 1978). Concentrations of ATP as low as 50 uM inhibit Tdt activity nearly completely, even if dNTP substrate levels in the reaction are increased 20-fold. The inhibition seems to be mediated by the blockage of the deoxyribonucleoside triphosphate binding site on the enzyme, rather than through a dead-end incorporation of the ribonucleoside triphosphate residue onto the primer terminus (Modak, 1978). Some workers had previously demonstrated that a few ribonucleotides could be incorporated onto DNA primers (Roychoudhury, 1972), but this ATP inhibition is not alleviated by the addition of excess primer. The inhibition is, however, slowly eliminated in the presence of a large excess of substrate (dGTP, for example). Current biochemical studies of terminal transferase are aimed at understanding the nature and structure of the functional DNA-binding site

of the enzyme, as well as defining the requirements for optimal nucleotide incorporation (Robbins et al., 1987).

Terminal Transferase Assays

There are presently three assays available for terminal deoxynucleotidyl transferase. The oldest and most refined is the enzymatic activity assay. The availability of antibodies specific for the protein led to both the immunofluorescence and immunoprecipitation assays. More recently, the isolation of nucleic acid probes for the Tdt gene product has enabled workers to monitor expression of the enzyme at a sensitive molecular level. Each of the assays has specific advantages and disadvantages, depending upon the application and source of Tdt.

The activity assay for terminal transferase was originally utilized to discern the enzyme from previously defined eukaryotic polymerases. It is the most definitive assay currently utilized, since an enzyme is defined primarily by its activity. The presence of an antibody-precipitated polypeptide or even a nucleic acid gene product is suggestive at best, until some consequence of the protein production can be observed. The Tdt addition reaction was originally defined for calf thymus enzyme,

and consisted of 0.03 mM initiator oligodeoxynucleotide (d(pTpTpT)), 8 mM $MgCl_2$, 1 mM ^{14}C-dATP, and purified enzyme, all in 40 mM potassium cacodylate buffer. Polymerization was monitored by scintillation counting of trichloroacetic acid (TCA) precipitable nucleic acid, with one unit of Tdt activity defined as the incorporation of one nanomole of labelled nucleotide in 1 hr at 35°C (Bollum, 1968). Since then, many modifications have been employed, including the use of crude enzyme from tissues or cell pellets (Coleman, 1977a). Optimal divalent cations and concentrations have been defined for Tdt from several species, and suitable oligonucleotide initiators and nucleoside triphosphate substrates have been determined (Coleman, 1977b). The use of crude lysates made for a more rapid assay, yet the most serious problem in the use of activity assays remains the lack of sensitivity. When dealing with whole thymuses or cells containing high levels of the enzyme, this is not a problem, yet enzyme activities less than 0.2 units are often undetectable.

The significance of low levels of terminal transferase cannot be ascertained until an _in vivo_ function for the enzyme is assigned; yet with current speculation favoring a somatic mutator activity (see below), the addition of just a few nucleotides per genome could be

crucial. For example, the addition of twenty bases by Tdt, in a particular region of a mammalian genome in one hour (plausible, especially with a 12 hr cell doubling time) could be the result of a Tdt activity level of approximately 3.3×10^{-15} units, or 3.3×10^{-7} units/10^8 cells, well below the sensitivity of current activity assays. A full unit of activity measured in a lysate from 10^8 cells would represent an addition of approximately 6×10^6 bases per genome per cell division. Of course such an addition in vivo would be difficult to imagine, yet many workers regard Tdt levels lower than 1 unit per 10^8 cells with skepticism. If in vitro activity is an accurate reflection of an in vivo event, one would have to reconcile high cellular Tdt activity with the maintenance of cell viability. In order to explain the absence of deleterious insertions, one would have to postulate some mechanism which either prevents nicking activities in a Tdt-containing cell or somehow renders 3'-OH groups inaccessible to terminal transferase. Such a proposal has not been suggested.

It is also possible that in some cases disruption of the cell could contribute to proteolysis, thus lowering apparent activities. The addition of reducing agents (1 mM 2-mercaptoethanol), a standard practice in the preparation of cell lysates, has been reported to inhibit

enzyme activity as much as 50 per cent (Jacobs and Hofert, 1983).

The activity assay for terminal transferase is important in providing a functional relevance for the protein, yet is hampered by a lack of sensitivity, the possibility of proteolytic degradation of the enzyme during preparation, and the usual arguments involved in ascribing in vivo significance to an in vitro activity.

The availability of purified calf terminal transferase enabled workers to elicit specific antibodies in rabbits, but only through repeated injections of a cross-linked form (Bollum, 1975). More recently, the production of monoclonal antibodies to Tdt has provided some measure of standardizations between laboratories (Bollum et al., 1984). These antibodies provided the reagents needed for immunological assays, namely immunofluorescence, immunoprecipitation, and radioimmunoassay, and have even been used to establish a conservation of structure of transferase peptides (Bollum et al., 1981).

The immunofluorescence assay derives its specificity from the antibody-enzyme reaction in permeabilized, cytocentrifuged preparations, and is visualized by the binding of a fluorescein conjugated second antibody. The assay was originally used to localize the sub-cellular distribution of the enzyme (Goldschneider et al., 1977)

and to screen cell populations quickly for Tdt positivity (Gregoire et al., 1979). The advantages of such an assay are the increased sensitivity (ideally, single cells should be assayable) and relative rapidity of the assay. The immunofluorescence assays are used routinely in the hematology clinics for leukemic cell classification. The suitability for screening leukemic samples is evident, since these cells generally are either high Tdt producers (relative to normal circulating homologues) or negative for the enzyme. The disadvantages of the assay include an inherent ambiguity present in fluorescent visualization, especially at low intracellular enzyme concentrations, and the fact that one is dealing with an immunologic specificity rather than an activity. The presence of the immunodominant moieties of the enzyme does not ensure the presence of functional Tdt. Ideally, a combination of both immunological and functional assays should be utilized to confirm a questionable result.

The enzyme immunoassay (ELISA) for Tdt differs from the immunofluorescence assay only in its mode of visualzation. It is a solid phase assay and is visualized by a colorimetric enzymatic reaction on the secondary antibody, as opposed to the fluorescent tag. The most recent commercially available kit (Abbott Laboratories) has previously been determined to be more sensitive, more

reproducible, and more stable than the immunofluorescent assays (Coleman et al., 1985). The use of an assay independent of radioisotopes (used in a radioimmunoassay) has always been preferred in a clinical setting, yet all of these immunological assays are only as good as the antibody used as the reagent.

The immunoprecipitation assay has been previously used to monitor biosynthesis of Tdt (Silverstone et al., 1980; Siden et al., 1985), yet is hampered by a dependence upon autoradiographic visualization. The assay depends upon the precipitation and visualization of in vivo radiolabelled proteins. The limits of detection have been appropriate and acceptable in these studies, yet it is difficult to classify a cell line as being Tdt^- using this criterion alone. Also, substantial background problems can be encountered, making detection of low levels of the enzyme difficult.

The most recently developed assay technique for terminal transferase is one that has not been fully exploited yet, owing to the sophistication of the technology required and the arduous nature of the sample preparation. The value of the cloned Tdt genes (Landau et al., 1984; Peterson et al., 1984) lies mainly in studies pertaining to the assigning of a role to the enzyme and determining its ontogeny and regulation. In

these cases, the presence of extremely low levels could be interpreted as significant and increase our awareness of the distribution of Tdt. The nucleic acid assays, which are the most sensitive currently available, are limited by the radioactive detection methods and by overinterpretation on the part of the investigator. The northern blot analysis, in which specific RNAs can be detected by hybridization to radiolabelled DNA probes, only provides evidence that the Tdt gene is being transcribed in the cells examined. No information regarding enzyme activity, or even Tdt protein production is obtained. Until the level of regulation of the gene is definitively ascertained, it remains to be seen whether this assay can be utilized quantitatively to yield meaningful results regarding Tdt activity. Its usefulness as a quantitative assay for clinical use is further limited by the presence of a basal level of Tdt mRNA in normal cells (Wolf et al., 1987). The nucleic acid probes do provide for a new avenue of creative experimentation aimed at discerning the role of Tdt. Researchers are currently attempting to put the Tdt gene into non-expressing cell lines at early stages of lymphoid differentiation and investigating the effects of its expression. The first steps in such enterprising studies have already been reported. Workers in F.J.

Bollum's laboratory have recently reported both immunoprecipitable protein production and enzymatic activity of a human terminal transferase cDNA clone in Escherichia coli (Peterson et al., 1985). Eukaryotic expression and activity of an exogenous Tdt gene has also recently been reported (Landau et al., 1987).

Ontogeny of Tdt^+ Cells

The unusual localization of Tdt in the lymphoid organs during ontogeny and differentiation is suggestive of a role in the development of the mature immune system. In addition, the presence of the enzyme in tumor cells derived from leukemias and lymphomas, themselves models of distinct stages of T and B lymohocyte development, has prompted investigators to examine the ontogeny of Tdt^+ cells, thereby clarifying the enzyme's role in lymphocyte maturation. Two approaches to such a classification have been utilized. The most straightforward series of experiments has involved the isolation of cells from various lymphoid organs during development and the correlation of cell surface markers with the presence of Tdt. Tumor cells of the lymphoid series have also been examined in this manner. Another approach to mapping Tdt^+ cells in the lymphocyte maturational scheme has been

to develop culture conditions that favor the growth of Tdt^+ cells. The surface marker studies, while a more convenient and rapid classification system, reveal little about the developmental potential of the Tdt^+ cells. The *in vitro* culture experiments allow for analysis of factors that favor Tdt-containing cell proliferation, but represent a rather artificial system. It is difficult to draw relevant *in vivo* correlates in such studies owing largely to the complexity of hormonal and cellular interactions not readily amenable to duplication in culture.

Early studies (Chang, 1971; Kung et al., 1975, 1976) established the lymphoid restriction of Tdt, and further provided circumstantial evidence that the cells containing the enzyme were thymocyte precursors (based upon cell surface markers). Silverstone et al. (1976) investigated the nature of the bone marrow population of Tdt^+ cells in the mouse by inducing the expression of the Thy-1 antigen (with thymopoietin) in bone marrow cells, selectively killing cells expressing the marker (by cytotoxic antibody treatment), and assaying Tdt activity in the remaining cells. They found that approximately 60% of the Tdt activity was destroyed by this procedure, and concluded that a significant portion of Tdt^+ cells in the murine marrow must already be committed to the T-cell pathway. Goldschneider et al. (1977) used immuno-

fluorescence and localized Tdt to the cortical regions of the murine thymus. This finding has had ample confirmation by other workers using antigenic analyses, cortisone sensitivity, peanut lectin binding, and size and buoyant density studies (reviewed in Goldschneider, 1982). The group further described the intracellular pattern of fluorescence as cytoplasmic, stating that no Tdt was detected in the nuclei of Tdt^+ thymocytes. This finding has been modified somewhat, in that the enzyme is predominantly detected in the nucleus and nuclear membrane, except during mitosis, when nuclear Tdt is released into the cytoplasm (Goldschneider, 1982). Gregoire and co-workers (1979) examined the ontogeny of Tdt^+ cell populations in hematopoietic tissues of rodents using immunofluorescence. In mice, Tdt^+ cells were first detected in fetal thymus at day 17 of gestation, peaking at birth. No other fetal cell populations were found to contain the enzyme. Another smaller wave of Tdt^+ cells was seen in hematopoietic tissues, such as the spleen and bone marrow, but not until after birth. The experimenters were able to conclude that this second wave was probably not of thymic origin, since normal numbers of Tdt^+ bone marrow and spleen cells were found in both neonatally thymectomized rats and congenitally athymic mice. Another important finding in this study was the

observation that at day 16 of gestation, an accumulation of Tdt^- cells was observed in the rodent thymus. This suggested that there is a stimulatory signal for Tdt expression in progenitor cells of the thymus (and probably other organs), and that the observed transferase positive cells arise *in situ*, rather than due to the influx of an already positive circulating population. Organ cultures display the same phenomena, supporting this idea. This work also serves to introduce an important caveat to be remembered when interpreting immunofluorescence data. Although neonatally thymectomized and congenitally athymic rodents had normal numbers of Tdt^+ cells in bone marrow, the specific activity of the enzyme was roughly one-half of normal values.

The analysis of Tdt^+ cells in humans is complicated by both ethical considerations and the possibility of immunologic abnormalities as a function of disease in biopsied samples. Nonetheless, Deibel and associates (1983) obtained thymic samples from 36 cardiac surgery patients of various ages with clinically normal immune function. Partial thymectomies are often performed in such cases to facilitate adequate heart exposure. Five fetal samples (anatomically normal spontaneous abortions) were also assayed for Tdt activity. The enzyme was

barely detectable in prenatal samples, low at birth, and highest between 10 and 40 months of age. This finding contrasts the high fetal Tdt levels observed in other mammalian systems (Chang, 1971). The total protein content of the thymic tissue showed no variance with age, so reported Tdt activities were valid activity estimates for the whole organ. The workers examined thymic architecture to ensure normalcy, in addition to assaying several other non-age related thymic enzymes (including adenosine kinase, adenosine deaminase, and 5'-nucleotidase) for specific activity standardization.

There have been several reports of enriched T cell progenitor populations that are inducible for Tdt, but very few examples of B cell lineage Tdt induction. These cell population studies were designed primarily to study the origins and developmental potentials of Tdt^+ cells, and to attempt to "place" these precursors ontogenetically (as described previously). Few projects were designed to ascertain the cascade of signals responsible for Tdt gene activation at the molecular level.

Many workers have confined their efforts to the identification of thymic factors responsible for Tdt expression. Such an emphasis upon the role of the thymus is obvious when one considers the tissue distribution of the enzyme. Previous work revealed that Tdt is asso-

ciated with a small bone marrow subpopulation (1-5% of total bone marrow cells), and that relatively low Tdt levels are seen in bone marrow from NIH Swiss nude mice (Silverstone et al., 1976). This group later concluded that Tdt can be synthesized by marrow cells, and probably does not come from Tdt^+ cells that migrate into the marrow (Silverstone et al., 1980). Pazmino and associates (1978) presented data that support the notion of Tdt bone marrow expression mediated by a regulatory molecule secreted by the thymus. They showed that Tdt induction in both congenitally athymic and surgically thymectomized mice is achieved *in vivo* upon injections of thymosin (fraction 5). This induction was seen *in vitro* as well, that suggests a direct hormone-like effect on the target cells, rather than indirect mediation through accessory cells. In addition, the effect was inhibitable *in vitro* by the addition of actinomycin D, revealing the need for active RNA synthesis. Low levels of thymosin have also been shown to induce a few other more mature T cell markers, including theta antigen (Basch and Goldstein, 1974) and Lyt expression (Goldschneider et al., 1981) in bone marrow-derived thymic progenitor cells.

There are only a few long-term systems reported in the literature that have been used to selectively

generate primary lymphoid cells in the absence of CFU-S. The Whitlock system (Whitlock and Witte, 1982) preferentially generates pre-B cells in vitro, while the system established in Goldschneider's laboratory (Hayashi et al., 1984) is specific for Tdt^+ cells. Both systems share several features, including modification of the existing Dexter culture system using adherent murine bone marrow cells, the use of fetal calf serum as a supplement (preferably with low corticosteroid levels), and similar incubation conditions (37^oC). The standard Dexter system was designed to generate CFU-S and GM-CFC cells, and utilized horse serum, hydrocortisone supplements, and 33^oC incubation. These in vitro culturing experiments arose out of a need to go beyond the use of hybridomas or transformed fetal cell lines, which proved invaluable to the study of allelic exclusion and Ig gene rearrangements. The culturing of normal cells allows one to study both progenitor cell populations and differentiated progeny. This is particularly useful in looking at the ontogeny of Tdt enzyme producing cells, and the results obtained from the two laboratories proved complementary. It is interesting to note the differences in the phenotypes of the cells obtained in each system, particularly amongst the Tdt^+ cells.

Hayashi et al. (1984) reported the development of an in vitro system for generating Tdt^+ bone marrow cells. Previous work by the same group relied upon modified Dexter cultures to generate murine Tdt^+ cells (1-2%) from bone marrow cells. This system also produced colony-forming unit spleen (CFU-s), erythroid, and myeloid precursor cells (Schrader et al, 1979). This newer system relied upon xenogeneic murine bone marrow feeder cells that selectively generated only undifferentiated Tdt-containing lymphoblasts from rat bone marrow primary cultures. The addition of a serum supplement that was extremely low in cortisol was also required. These Tdt^+ cells were characterized as being T-cell progenitors, based upon sensitivity to various steroids, morphology, and pattern of Tdt fluorescence. Some of the published criteria (e.g., no surface or cytoplasmic Ig detected), however, leave much room for contention. This group favors a pre-T cell origin for the progenitor Tdt^+ cell.

Experiments performed in the laboratory of Owen Witte have led to the development of long term cultures of both murine bone marrow and fetal liver cells (Whitlock and Witte, 1982; Denis et al., 1984). While the bone marrow cultures generate a mixture of B cell precursors, pre-B cells, and IgM-expressing B cells, the fetal liver system retains a B cell precursor phenotype even after several

months in culture (maintenance of cell populations containing an unrearranged immunoglobulin locus). An analysis of some of the bone marrow cultures revealed that 7 out of 11 clones of Abelson virus-transformed murine pre-B cells expressed Tdt, supporting the belief that most Tdt^+ cells (and even the cells described by Hayashi et al. above) may represent primitive members of the pre-B lineage. Hayashi et al. countered by citing data from Witte's lab that revealed that only 1 out of 6 transformed pre-B cell lines in their system were Tdt^+ when the nontransformed precursors were cloned prior to infection with Abelson virus (Whitlock et al., 1983). Further, the one Tdt^+ clone originated from a precursor that was unrearranged at the mu heavy chain locus. Rather than argue which of the two systems is more analogous to *in vivo* differentiation, the groups should perhaps look for a pleiotropic precursor line capable of differentiation along either pathway.

A final approach to the ontogenetic study of Tdt expression has been undertaken by Silverstone and many coworkers (1978). This group has relied upon the supposition that tumor cells represent defined steps in a normal developmental program. Tumor cells have long been used successfully as models of distinct stages in B and T lymphocyte development, and proved both useful and

accurate in the analyses of immunoglobulin gene rearrangement. Tumor cell phenotypes representing nearly all stages of B cell differentiation have been validated by the identification of their normal cell analogs. The fact that tumor cells represent "frozen" or static counterparts to normal cell stages does not preclude the induction of pre-programmed gene expression either spontaneously or upon exposure to a suitable inducing agent. Such a phenomenon has been demonstrated with malignant teratocarcinoma cells, which are capable of giving rise to all of the normal cell sets for which they are programmed (Illmensee and Mintz, 1976). The large number of genes induced by cAMP (described below) have also been shown to be linked to a developmental "program."

The tumor cell studies have produced a model of Tdt expression in which only the earliest identifiable cells from the B and T cell lineages produce the enzyme. Neither the presumptive multipotential CFU-S nor the early Ly1-4, Sc-1 pro-lymphocyte express Tdt, yet the next defined stages of differentiation do (Lyb-2, Ly1-4, and Sc-1 cells in the B cell lineage, and Thy-1, TL, Lyt1,2 in T cells), as reported by Silverstone and coworkers (1978). No other cells described by this group have been shown to possess Tdt activity. Many other

groups since then have developed more monoclonal antibodies designed to further delineate Tdt^+ cells (Goldschneider et al., 1981; Bodger et al., 1981), but all agree on the fact that Tdt expression is restricted to a very early and transient cell differentiative stage.

There is a noteworthy and provocative finding in the ontogeny of Tdt appearance in amphibians. Studies done in Xenopus embryos revealed that Tdt expression preceded the development of the thymus by several days. In fact, Tdt expression was detectable within two days post-fertilization, just one day after the first immunoglobulin heavy chain mRNA transcripts appear (Brown, 1981). Interesting, too, is the fact that the first thymic derived lymphocytes are not apparent until day 6 post-fertilization. The author concluded that Tdt expression is independent of the appearance of an intact thymus. It would be interesting to study the immunoglobulin transcripts of Xenopus (coding for both the 19S and 7S Ig proteins) to see if any regions of variability which may be attributable to the action of Tdt are seen. Also, it would be simple and enlightening to determine whether this early Tdt expression is derived from newly synthesized fetal mRNA, or maternal stored transcripts. It is fascinating to speculate as to the purpose of this very early Tdt expression.

ALL and Tdt

The earliest association between Tdt and a disease state was made by McCaffrey and Baltimore (1972) and was erroneously reported as reverse transcriptase activity. Following the discovery of reverse transcriptase in retroviruses, a concerted effort was made to isolate the enzyme from murine and human leukemic cells. Such a finding would have supported a generalized viral origin of human leukemogenesis. Both Moloney leukemia virus-infected murine embryonic bone marrow fibroblasts and human leukemic cells (clinical isolates) were assayed for reverse transcriptase, in an *in vitro* assay using a poly (C) template and an oligo (dG) primer. Synthesis of poly (dG) was observed in both cases and attributed to this unique virus-specific enzyme. Careful "leaving out" experiments later revealed that poly (dG) was synthesized in the absence of either the primer or the template (McCaffrey et al., 1973a). Enzyme purification and analysis revealed the template-independent polymerization to be the result of terminal transferase activity.

The investigations resulting from the early reverse transcriptase assays led to the characterization of cell lysates from three different clinical isolates: an acute

lymphocytic leukemia (ALL), a lymphosarcoma, and an acute myeloblastic leukemia (AML) (McCaffrey et al., 1973a). Meticulous protein purification and assaying techniques revealed that only the ALL cells contained measurable Tdt activity. All leukemic cell extracts, incidentally, were negative for reverse transcriptase. The authors suggested that the leukemic event in ALL represented a block in normal thymocyte development, and that Tdt could prove to be a useful marker in identifying leukemic cell origin. At the time of publication, however, the presence of Tdt in mammalian bone marrow cells had not yet been reported (due to the insensitivity of available Tdt assays).

Since these pioneering studies, Tdt positivity has been seen in lymphoblasts from 90-95% of all ALL cases, making the enzyme a diagnostic marker of the disease (McCaffrey et al., 1975; Kurec and Davey, 1981). Less than 5% of blasts isolated from non-ALL cases are Tdt^+.

Extensive correlative analyses have been performed on bone marrow lymphoblasts from patients with diagnosed childhood acute leukemias, in order to ascertain the clinical utility of Tdt determinations (Kalwinsky et al., 1981). Morphologically diagnosed ALL blasts revealed Tdt levels ranging from 1-1500 U/10^8 cells (median=90 U), whereas control, non-leukemic samples ranged from 1-220

$U/10^8$ cells (median=2.6 U). The authors were even able to derive a probability equation that will predict the chances of a new patient with undefined acute leukemia having ALL, based solely upon the initial Tdt value. They concluded that a patient presenting with blast Tdt values of 20 U or more at diagnosis has a 90% probability of having ALL. Due to the cost and time involved in quantitative Tdt assaying, however, most institutions today rely upon the qualitative immunofluorescence assay as an ALL diagnostic tool.

One of the more interesting aspects of the association between Tdt and leukemic cells is the possibility of enzyme inhibitor chemotherapy. Therapeutic uses of selective inhibitors of enzyme function have been postulated and tested for many types of leukemias and enzymes, as reviewed by Coleman (1983). Current chemotherapies for lymphocytic leukemias rely upon agents such as vincristine, prednisone, or nucleoside analogs, which affect normal cellular metabolic processes such as tubulin elongation, or nucleic acid and protein synthesis (Kirshner and Preisler, 1985). It has been reported that Tdt^+ leukemias are more apt to respond to such therapy than Tdt^- cases (Marks et al., 1978). The use of such compounds takes advantage of the relatively high metabolic and replicative rate of leukemic

cells relative to normal cell populations. The availability of agents that are specific for disease-associated enzymes may significantly reduce the toxic effects on normal cell populations lacking these enzymes. Therapeutic potential, however, is dependent upon a causal relationship between specific enzymes and a disease state, as opposed to simply the association which currently exists.

McCaffrey and coworkers have identified several derivitives of 6-anilinouracil which can specifically inhibit Tdt activity *in vitro*, with little or no effect upon polymerase alpha, beta, gamma, or AMV reverse transcriptase activity (McCaffrey et al., 1983). In addition, these inhibitor compounds have been shown to inhibit the growth of only Tdt^+ cells *in vitro*, as well as normal Tdt^+ cells *in vivo*. Preliminary *in vivo* acute toxicity studies of the compounds were negative, and decreases in thymic weight were attributed to specific cytotoxicity of normal Tdt^+ cells (McCaffrey, R. personal communication). These data may imply that Tdt is vital in maintaining the viability of these cells. Presumably, the normal Tdt^+ cell population of the thymus would be subject to self-renewal following remission of the leukemic state, thereby reducing the risk of irreversible immune system dysfunction.

Rusquet et al. (1985) examined the effects of various antileukemic compounds on the enzymatic activity of Tdt purified from normal thymocytes and Tdt^+ ALL lymphoblasts. They found that most agents tested effectively inhibited Tdt from both sources to an equal degree, while a few agents preferentially inhibited either the thymic enzyme (adriamycin) or the leukemic enzyme (vincristine).

The link established between acute lymphocytic leukemia and Tdt is well documented. Prognostic information gained by the assessment of tumor cell Tdt phenotypes is valuable enough to routinely order the enzyme assay in all possible ALL cases. The chemotherapeutic potential of Tdt inhibition is just beginning to be investigated, and if the preliminary data are an indicator, then selective enzyme inhibitor therapy may represent the next generation of treatment modalities in the battle against cancer.

The Role of Terminal Transferase

Despite current knowledge of the *in vitro* activity of Tdt, its tissue and organ distribution, and the high correlation with various disease states, the *in vivo* function of the enzyme has remained a mystery. In 1974, Baltimore hypothesized that Tdt could function as a

somatic mutator of immunoglobulin (Ig) variable regions (Baltimore, 1974). Non-heritable somatic changes in the primary germ-line DNA sequence for the variable region of the antibody polypeptide have been hypothesized as a source of the seemingly infinite combining-site repertoire of mammalian immune systems. The model that Baltimore described involved single-strand breakage in the hypervariable region of the antibody genes, followed by Tdt mediated "random" base incorporation, and repair by normal host polymerases and ligases. Inherent in the model is a site specific endonuclease that can act at hypervariable V regions. The hypothesis stood the test of time, until around 1981, when the molecular biology of the Ig locus was advanced enough to allow careful scrutinization of the theory.

An extraordinarily high frequency of somatic point mutations had been noted in assembled variable region genes (Weigert et al., 1970; Kim et al., 1981). Studies carried out in several laboratories, however, determined that this somatic point mutation occurs late in B-lymphocyte development, at a point when the cells no longer contain detectable Tdt (Kung et al., 1975; Gearhardt et al., 1981). In fact, the point mutations actually accumulate with the class switching event characteristic of B cell maturation. At this point, even

Baltimore (1981) expressed some doubt about Tdt and V-region hypermutation.

Recent data have revealed that mutations of the Ig locus are not restricted to the protein coding region; somatic mutations of germ line sequences are observed in non-coding introns near rearranged V-J segments (Kim et al., 1981; Gearhart and Bogenhagen, 1983). The earliest evidence of somatic mutation has been observed in pre-B cells (Ziegler et al., 1984; Wabl et al., 1985). The fact that detectable, albeit low, levels of Tdt can be demonstrated in transformed pre-B cell lines lent credence to Baltimore's original theory, however the presence of high Tdt levels in thymocytes precluded an Ig exclusive mutation process. Recent data from several laboratories (Barth et al., 1985; Sim, 1985) have revealed evidence of somatic point mutation in the T cell receptor locus, so some still regard the enzyme as a generalized lymphoid cell receptor point mutator. Other groups, however, have not seen evidence of point mutations in the T cell receptor, and contradict these interpretations (Ikuta et al., 1985; Klein et al., 1987). Recent studies surrounding the "random" nature of Tdt-mediated polymerization have helped to advance the currently favored role of Tdt in lymphoid differentiation.

Since Tdt does not respond to template direction, and will incorporate all four nucleotides in a synthesis reaction, it has long been regarded as an "error-prone," or random, polymerase. Recent studies, however, have discounted the seemingly random nature of Tdt catalysis products. Basu and coworkers (1983) examined the control of Tdt synthetic activity with respect to substrate nucleotide concentrations, nature of the priming DNA, and the presence of accessory proteins. Interestingly, they found that the composition of the DNA products made by Tdt when equimolar concentrations of all four deoxynucleoside triphosphates were present is uniquely dGMP-rich. This observation is independent of the priming DNA used in the reaction (single stranded, double stranded, or chromatin), and was seen when either Mn^{2+} or Mg^{2+} was provided as the divalent cation. Such a finding is not wholly unexpected. As early as 1973, McCaffrey and coworkers (1973b) reported that dGTP routinely yielded the highest incorporation levels of any deoxynucleotide substrate, regardless of the composition of the homopolymeric primer used.

Recently, a strong correlation has been noted between Tdt levels and the presence of "N regions," small streches of DNA located between both the V and the D region, and the D and the J region of a rearranged

immunoglobulin heavy chain locus (Alt and Baltimore, 1982; Desiderio et al., 1984). These N regions are thought to arise de novo, since no germ line rearrangement can account for their presence. In addition, the N regions are unusually rich in dG residues, a preferred nucleotide which Tdt incorporates in in vitro assays (Basu et al., 1983). N-regions have also been reported in the T cell receptor locus (Barth et al., 1985; Goverman et al., 1985; Klein et al., 1987).

Landau et al. (1987) have performed one of the most revealing experiments linking Tdt expression with N-region insertion in Ig junctional sites. They inserted a functional Tdt gene into a pre-B cell line that rearranges kappa light chain genes, but normally lacks Tdt (as assayed by northern blot analysis of Tdt mRNA levels). The expressed Tdt gene was introduced via a retroviral expression vector. The cells were then superinfected with a virus containing a kappa Ig light chain construct previously shown to undergo rearrangement in suitable cells. Analysis of the input kappa construct after several rounds of cell division revealed that the presence of Tdt in the cell increased the frequency of N-region insertion by at least 5-fold relative to cells lacking the enzyme. There were several problems with the study, including the use of light chain junctions in the

assay (the majority of reported N-regions have been found in the heavy chain locus), plus the fact that the group dismissed those clones that failed to insert N-regions and showed no detectable Tdt in the cell (despite the presence of the inserted Tdt gene). Yet the fact that 10% of the constructs in control cells without the inserted Tdt gene contained N-regions was explained by sub-detectable (yet perhaps active) levels of the enzyme. Further, the majority of N-regions analyzed were only 1-2 bases in length--much smaller than N-regions seen in heavy chain genes. The lengths of the N-regions observed did not correlate with the amount of Tdt expressed in the cell, although the presence or absence of the N-regions did.

The study does represent an improvement over previous studies (Yancopoulos et al., 1986) that relied on independent cell lines that differed in Tdt levels (and perhaps other gene products, as well).

Schroeder et al. (1987) recently reported the observation of N regions in the human system. These workers cloned segments of the human immunoglobulin locus from fetal liver cells at 130 days of gestation, in order to examine V_H repertoires. Nearly all of the cDNA clones isolated and sequenced were found to contain G-C rich N regions, which varied from 12-34 bases. This hetero-

geneity, they conclude, is responsible for a great deal of variation seen around the third hypervariable region of the fetal antibody heavy chains.

An interesting series of experiments has been undertaken by a group that has been examining the actions and physiological roles of various eukaryotic DNA polymerases. They have recently developed in vitro assays to demonstrate the type of DNA rearrangements that may be mediated by Tdt (Kunkel et al., 1986). Their assays consisted of both a ØX174 replication system (to measure fidelity) and an M13mp2 forward mutation assay (to measure the spectrum of mutations arising). More recently, this group has looked at misincorporation frequencies using poly [d(A-T)] templates and nearest neighbor product analyses (Snow et al, 1987). The ØX system is quite sensitive to single base substitutions, and utilized a phage containing an amber mutation, copied with beta polymerase plus or minus Tdt, in a reversion assay. The results in these experiments revealed a rather high basal level of misincorporation with pol B, which is only slightly heightened by the addition of Tdt to the reaction. This slight increase was abolished with simultaneous addition of anti-Tdt antiserum. It would have been illuminating to examine the actual transfection data, in order to assess the degree of lethal mutations

produced in the presence of Tdt. The M13mp2 assay detects *in vitro* errors produced during synthesis, using a gapped double stranded circular molecule (with the single-stranded gap containing the lacZ gene cloned in M13mp2 DNA). This gap is filled *in vitro* by exogenously added polymerase, and the product is used to infect cells to score alpha complementation mutants. Mutant plaques are colorless (or at least lighter) than plaques made by wild-type M13. Using chicken polymerase beta or alpha, or human hepatoma polymerase beta, a significant increase in the mutation frequency is observed in the presence of Tdt. Tdt alone also proved to be highly mutagenic in these assays. All mutants that were analyzed (by sequencing) revealed that Tdt was responsible for "complex" mutations, as opposed to point mutations attributed to the alpha or beta polymerases. In twelve mutations mediated by Tdt, it was determined that the enzyme resulted in the loss of large stretches of template DNA sequences, with the insertion of random new sequences. Two mutants were seen to have an equal number of inserted and randomly replaced nucleotides, but this type of event was clearly rare. The sequence analysis of the mutants produced by Tdt alone reveals the characteristic preference of the enzyme for inserting deoxy-

guanosine residues (guanine comprised 68% of the bases inserted).

Based upon these studies, Snow and coworkers have concluded that Tdt does not catalyze the majority of scattered single-base mutations commonly seen during antibody gene differentiation. They attribute these changes to some other error-prone DNA synthetic process, perhaps mediated through DNA polymerase B. They do, however, provide evidence that supports the role of Tdt as a component of a junctional recombinase process, and perhaps integral to the formation of N-region diversity.

Several distinct models for site specific somatic mutation have been proposed (for example, Alt and Baltimore, 1982; Selsing and Storb, 1981), yet all involve an error-prone polymerization step followed by mismatch repair synthesis. Terminal transferase has repeatedly been implicated as the mutator polymerase.

The model put forth by Kunkel et al. (1986) included a five step scheme for Tdt-mediated generation of N-regions. Their model, which is the most recently proposed, relies upon large deletions generated from the joining and rejoining of two distant DNA sequences, the insertion of new dG rich sequences (by Tdt), and perhaps additional, neighboring point mutations generated at the same time. Specifically, they propose (1) a dissociation

of DNA from the rearranging primer-template, followed by (2) the action of Tdt upon the free 3'-hydroxyl resulting in N-region formation. The DNA strands (primer and template) are then (3) reformed by imperfect hybridization, with ends being (4) subjected to extensive template-directed synthesis by DNA polymerase, and finally the resolution of the single-stranded Tdt-generated "bubble" into a newly synthesized daughter strand (containing additional bases generated by Tdt, and perhaps lacking some parental template sequences), and the unaffected parental strand. The model awaits rigorous testing.

Wysocki et al. (1986) investigated the junctional sequences in a large panel of monoclonal antibodies encoded by one particular V_H gene, in order to assess the in vivo predictions of Tdt-inserted N-regions. Examination of DNA from 33 anti-Ars (p-azophenylarsonate) hybridomas revealed preferential codon usage for a specific serine residue appearing at the V_H-D border. This serine residue, it was found, is essential for high affinity antigen binding. Their premise was that if template-independent de novo nucleotide addition was important, then any one of the six possible serine residues would be observed at this particular position. They concluded that the absence of other serine codons

precluded a role for Tdt, and suggested a sequence-specific origin for many V_H-D junctions. The data, while certainly valid, has not yet been extended to other systems. The forces in nature that dictate codon usage in such an essential region are not well understood. Perhaps, through some selective pressure, virgin cells expressing randomly inserted bases in this region were eliminated, or at least not stimulated.

Foa et al. (1987) studied Tdt expression in acute myeloid leukemia (AML) cells. McCaffrey et al. (1973b) examined a few AML cases and reported them as Tdt^-, but this is presumably due to the fact that only a small proportion (5-10%) of these cells express the enzyme. Foa and coworkers studied 52 AML cases, of which 10 were Tdt^+. The myeloid classification was based upon cytochemical and immunological criteria, together with an absence of T and B cell markers. Their data revealed a marked correlation between Tdt expression and Igh rearrangements in these cells. Eight of the ten J_H rearranged loci belonged to the Tdt^+ group, while only two of the 42 Tdt^- cases had some rearrangement. Interestingly, when the T cell beta and gamma receptors were examined, four out of the total 53 cases were rearranged, and these all were from the Tdt^+ group. The authors state that the evidence that both Igh and TCR-gamma rearrangements

positively correlate with Tdt expression favors a concept that a unique enzymatic machinery may be involved in B and T cell specific loci. Further, the findings support the view that Tdt expression may be coupled to the activity of a V-D-J specific recombinase (Yancopoulos et al., 1986). Relevant, too, is the finding that the pattern of Tdt expression seen in human thymus ontogeny has been shown to coincide with the increase seen in human immunoglobulin levels during maturation (Deibel et al., 1983).

Of considerable interest (though not pertinent to this discussion) is the finding of biphenotypy (lymphoid and myeloid) in many of these AML cases described by Foa et al. (1987). Greaves et al. (1986) have hypothesized that there is a certain degree of promiscuity in the normal process of hematopoietic differentiation, such that some markers, normally restricted to one lineage, may be transiently coexpressed.

An important area of concern, and one that has not been adequately addressed in the course of studying Tdt is that of the intracellular state of the enzyme. For a long time, there was some question as to whether or not the enzyme is even active *in vivo*. All studies previous to the series of Tdt-gene insertion experiments by Landau et al. (1987) had relied upon an assessment of Tdt

activity either in dialyzed crude lysates or purified preparations. Such analyses, while perhaps appropriate for comparison purposes, do not address the myriad of variables involved. Inhibitors, such as ATP, a normal and fluctuating intracellular component are effectively diluted, dialyzed, or purified to lower than normal levels.

The intracellular activity of Tdt is also of concern with respect to competition with other DNA polymerases. The danger of having a potentially mutagentic polymerase acting upon growing replication forks or chromosome termini is too large to disregard. In a Tdt positive cortical thymocyte, for example, how is Tdt inhibited from acting upon any free DNA 3'-OH? The purified enzyme exhibits similar K_m values to eukaryotic alpha and beta polymerases (Kornberg, 1980; Deibel et al., 1980). The enzyme is not subject to cell cycle variations (Harrison et al., 1976), which eliminates an effective regulatory mechanism.

The possibility of post-translational regulation has not been extensively entertained in the literature. Fluctuations in ATP pool sizes may play a role in post-translational Tdt regulation, but this possibility has not been investigated. Protein phosphorylation, which has rapidly become a central explanation for cellular

functions ranging from growth inhibition to transformation and uncontrolled growth, is viewed by some as a control mechanism for Tdt activity (Elias et al. 1982). These authors report that NALM-6 (human lymphoblastic leukemia) cells contain phosphorylated Tdt (85% phosphoserine and 15% phosphothreonine). This is interesting, but may not be too relevant to normal cell physiology, owing to the variety of phosphorylating activities seen in transformed cells, and the failure of the authors to distinguish the tumor cell viral-mediated phosphorylation from other intracellular phosphorylation. The fact that Tdt activity is decreased upon incubation of purified calf Tdt with leukemic cell extracts is interesting. Elias and coworkers attributed this decreased activity to Tdt phosphorylation by cAMP-independent casein kinases found in leukemic cells, yet have not provided compelling evidence.

Cyclic AMP

Cyclic adenosine 3',5'-monophosphate (cyclic AMP; cAMP) has been implicated in a wide variety of regulatory processes in mammalian cells and microorganisms. The structure of cAMP was first described simultaneously in the laboratories of Roy Markham (Cook et al., 1957) and

Earl Sutherland (Sutherland and Rall, 1957). The recognition of its physiological role dates from investigations by Sutherland and his collegues into the mechanism of the hyperglycemic action of epinephrine and glucagon (Sutherland and Rall, 1960). Cyclic AMP was shown to be the factor responsible for phosphorylase activation (and subsequently glycogenolysis) in liver cells following incubation with glucagon or epinephrine (Sutherland, et. al. 1972). The activation of phosphorylase from the inactive form is mediated by phosphorylase kinase, upon which cAMP exerts its effects (Robison et al., 1968). The cAMP, they ascertained, was produced in a reaction mediated by the membrane enzyme adenylate cyclase, and dependent upon cellular ATP as a substrate. Since then, much work has revealed both the ubiquity of cAMP in living organisms, and the universality of its regulatory functions.

There are several conserved patterns of cAMP regulation between prokaryotes and eukaryotes. Among these is an increase in the cAMP concentrations in response to stress or membrane depolarization. In an extreme situation, either of these can stimulate the degradation of cellular reserves (carbohydrates, in liver cells, for example) and lead to an increase in the availability of ATP. In E. coli (and other prokaryotes),

the role of cAMP in cellular regulation is seen at the RNA transcriptional level, the major genetic regulatory process in prokaryotes. The availability of carbon containing compounds was found to affect cAMP levels, which in turn regulate a variety of operons, which are said to be catabolite repressible (Pall, 1984). A spectrum of biological effects in eukaryotes that are mediated through cAMP seem to proceed through a common series of events. These include the binding of an effector to the external cell membrane, signal transduction to membrane-associated adenylate cyclase, and subsequent activation of that enzyme, resulting in intracellular cAMP elevations. In eukaryotes, cyclic AMP has been shown to activate a cascade of protein kinases, which phosphorylate specific protein substrates, ultimately resulting in an alteration in their biological activity. The role of cAMP in cellular regulation is by no means universal. In certain mutants of the S49 (lymphoma) cell line, cAMP-dependent protein kinase is lacking, with no observable effects upon cellular growth (Gancedo et. al., 1985). While the function of cAMP as a second messenger, relaying extracellular signals to the interior of the cell, may be conserved through evolution, the complexity of the system in eukaryotes has undergone extensive modification. Both the spectrum of gene

products affected by cAMP, and their temporal expression reveal the importance of this small, yet important molecule in nature.

The intracellular level of cAMP at any given instant depends mainly upon the activity of two enzymes, adenylate cyclase and phosphodiesterase. Adenylate cyclase catalyzes the formation of cAMP from ATP in a reaction which requires magnesium (and liberates pyrophosphate) (Sutherland, et. al., 1962). Inactivation of cAMP is mediated by a specific phosphodiesterase, which catalyzes its hydrolysis to 5'-AMP (Butcher and Sutherland, 1962). Regulation of cAMP has been shown to occur by the exclusive modulation of one of these two enzymes by various agents.

Colchicine, as well as other substances that interfere with microtubule assembly (and inhibit DNA synthesis), increases intracellular cAMP levels, and potentiate the effects of hormones on cAMP levels in human leukocytes. The mechanism of action appears to be through an increase in the synthesis of cAMP, as opposed to a decreased rate of cAMP hydrolysis (Rudolph et. al., 1977). Other exogenous agents, such as dibutyryl cAMP (dbcAMP) used in this study, have also been shown to indirectly affect intracellular cAMP levels.

$N^6,O^{2'}$-Dibutyryl adenosine cyclic 3':5'-monophosphate, a derivitive of cAMP often used to mimic the actions of endogenous cAMP, has been previously studied in CHO cells by O'Neill and co-workers (1975). They found that the compound is partially hydrolyzed in the medium, taken up by the cell, and further metabolized to N^6-monobutyryl AMP, which accumulates. This product acts as an inhibitor of cyclic AMP phosphodiesterase, resulting in an increased intracellular cAMP level.

The concept of the "second messenger" (Robison et al., 1968), a hypothesis that explains cAMP mediation of hormonal and external stimulatory action, has found ample confirmation in many systems. The model regards hormones as first messengers, which travel from their cells of origin to cells of target tissues, and there stimulate the formation of a second messenger, such as cAMP. Within such a model, the adenylate cyclase system has a dual role--both as a discriminator to interpret environmental signals, and as a signal generator. The second messenger hypothesis was used as a prototypical model of lower eukaryotic hormone action by John Bonner and coworkers to explain the chemotactic aggregative response of slime mold amoebae (Dictyostelium discoideum), first observed in the late 1940's (Kessin, 1981). The effect

was shown to be developmentally regulated, and mediated through a cAMP (then known as acrasin) induced protein.

Mangiarotti et al. (1983) looked at transcription and mRNA stabilization in Dictyostelium. They concluded that exogenously added cAMP will supplant the need for cell-to-cell aggregation. cAMP added to disaggregated cells will restore the synthesis of developmental RNAs and also prevent their destabilization. The implication is that perhaps cAMP has a physiologic role in controlling transcription and mRNA stability in normal development.

In addition to a chemotactic response in amoeba of Dictyostelium, an increase in intracellular cAMP appears either to be required for, or to accelerate patterns in developmental gene expression. For example, Williams et al. (1980) did studies in the isolated nuceli of Dictyostelium and found that the addition of cAMP during the first few hours of development decreased transcription rates of the discoidin genes (carbohydrate binding proteins) while resulting in a premature accumulation of several mRNAs normally expressed late in development. Thus, the role of cAMP can be expanded to that of a modulator of gene expression, rather than simply an activator of protein modification. The cAMP

acts as a catalyst for the activation of a pre-ordained program of developmental gene expression.

The main cAMP-mediated system of regulation in eukaryotes, that of protein phosphorylation, was first noted in the late 1950's by Krebs and coworkers (Krebs et al., 1959). They determined that the activity of the rate limiting enzyme of glycogenolysis, glycogen phosphorylase, was regulated by the actions of phosphorylase kinase, itself an interconvertible enzyme whose activity depended upon its phosphorylation state. The study of protein phosphorylation was underway, yet it was not until the discovery of cAMP-dependent protein kinase (Walsh et al., 1968; Kuo and Greengard, 1969) that the field blossomed. The number of enzymes and other proteins whose activity is regulated by phosphorylation-dephosphorylation is enormous, leading one to believe that protein phosphorylation may well be the major regulatory mechanism by which intracellular metabolism is modified by extracellular stimuli.

Cyclic AMP has previously been shown to induce serine phosphorylation of $pp60^{src}$ increasing its kinase activity (Roth et al., 1983). In addition, cAMP stimulates serine and threonine phosphorylation of epidermal growth factor receptors solubilized from A431 cell membranes (Ghosh-Dastinar and Fox, 1984). Elevation

of intracellular cAMP levels increases the phosphorylation of the B subunit of insulin receptors in lymphoblasts by approximately two-fold, by an uncharacterized protein kinase (Stadtmauer and Rosen, 1986). These authors suggest that cAMP may attenuate the action of insulin by the alteration of the phosphorylation state of the insulin receptor.

An interesting finding, and one of particular relevance to this work is that in the analysis of discoidin mRNAs in Dictyostelium, which exhibit developmentally accelerated expression in the presence of exogenous cAMP, a considerable lag period between the time of cAMP addition and the onset of detectable discoidin mRNA accumulation was observed (Williams et al., 1980). This six hour lag period, according to these workers, demonstrates that some other conditions must be fulfilled before these genes can be expressed. Perhaps a cascade involving newly synthesized proteins is required, as opposed to the cAMP-dependent protein kinase systems utilized in intermediary metabolism of higher eukaryotes, which respond to physiologic stimuli rapidly. There are several other examples of temporally delayed gene expression after cAMP exposure.

Mendelson, et al. (1986) reported that the induction of pulmonary surfactant apoprotein during *in vitro* and *in*

vivo differentiation of rabbit lung cells was associated with an increase in specific mRNA content in the cell, and that this phenomenon was also brought about with the addition of cAMP analogues (including dbcAMP) and other agents shown to increase intracellular cAMP levels. The observed changes began at about 24 hrs post induction, and continued for several days in culture. This is another example of a "slower" molecular change mediated by cAMP.

Dibutyryl cAMP induces axon formation in murine neuroblastoma cells in vitro (Prasad and Hsie, 1971). Exposure of the cells to X-irradiation has been shown to do the same (Prasad, 1971), but these authors did not address a possible link between the two processes. The neuroblastoma cells used have an in vitro doubling time of about 24 hours, which is the same amount of culture time in the presence of dbcAMP required to demonstrate axonal growth. The peak effect of the cAMP was noted approximately 3 days post-addition. The increase in differentiation noted seemed to be at the expense of cell growth. The growth inhibition observed by dbcAMP was largely irreversible, since washing out the dbcAMP from the medium after prolonged incubation failed to return the cells to control cell growth kinetics. Thus, the workers consider this phenomenon to be an example of

irreversible differentiation. They go on to state that the inhibition of cell division is necessary, though not sufficient for cell differentiation, since other agents (such as sodium butyrate) inhibited cell growth, but failed to elicit axonal growth.

The number of eukaryotic genes regulated either directly or indirectly via cAMP modulation is immense. While the mechanisms of control are, for the most part, speculative, considerable effort is currently being expended in order to clarify these. It is informative to consider a few select examples of the systems known to be mediated through cAMP, in order to gain an appreciation for the diverse nature of the genes under the control of this ubiquitous agent.

One of the best characterized systems of cAMP-mediated gene regulation is that of the phospho-enolpyruvate carboxykinase (PEPCK) gene in the rat liver. Work done in R. Hanson's laboratory (Lamers et al., 1982; Meisner et al., 1983) has revealed that the PEPCK gene is regulated at the transcriptional level.

Previous work had shown that administration of dbcAMP to a glucose-fed rat rapidly increases the levels of cytosolic PEPCK mRNA, an effect that can be blocked by the simultaneous addition of either cordycepin or actino-mycin D. This group used a PEPCK cDNA probe and measured

transcription rates of the PEPCK gene in isolated rat hepatocyte nuclei. A four to seven-fold stimulation of PEPCK gene expression was noted after dbcAMP treatment of glucose-fed rats. The transcription was inhibited over 80% by refeeding starved rats with glucose. The researchers, using deletion mutants, next examined the promoter region (5' upstream) of the PEPCK gene, and localized the putative cAMP regulatory element within a 47 bp region, which is approximately 100 bp upstream of the transcription start site. At the heart of this sequence lies a 12 bp core sequence, which is significantly homologous to regions in other cAMP-regulated genes (Short et al., 1986), including rat preprosomatostatin, vasoactive intestinal polypeptide, and porcine plasminogen activator. In fact, in genes where these elements have been discovered, the elements were found to be situated in close proximity to cAMP regulatory sites.

There are several other genes in which expression has been linked to cAMP, including: lactate dehydrogenase, tyrosine aminotransferase, and prolactin. Prolactin, a product of the anterior pituitary gland, is responsible for regulation of lactation in pregnancy. Dopaminergic regulation of prolactin gene expression has previously been demonstrated to be mediated by cAMP. In the absence of dopamine, pituitary cells contain high levels

of cAMP that stimulate prolactin gene expression and lead to the accumulation of prolactin mRNA. Dopaminergic treatments inhibit pituitary adenylate cyclase and decrease intracellular cAMP levels, leading to decreases in prolactin mRNA concentrations. The addition of exogenous cAMP analogs rapidly increases prolactin mRNA concentrations, implying that the ability of cAMP to alter prolactin RNA levels involves effects at the transcriptional level. Treatment of pituitary cells with ergocryptine, a dopamine agonist, will cause a rapid inhibition of prolactin gene transcription. If a cyclic AMP analog is then added to these cells , then a rapid stimulation of prolactin gene expression ensues (Maurer, 1981). This study examined prolactin mRNA accumulation primarily by monitoring in vitro run-off transcripts in isolated pituitary cell nuclei, as a function of total RNA transcription. Along with an enhancement of prolactin gene transcription, cAMP has been shown to phosphorylate a unique chromatin-associated protein. It has been suggested that cAMP may act through an alteration of the phosphorylation state of a nuclear protein, or by the formation of a receptor-cAMP complex that can directly interact with a DNA regulatory site. There is precedence for the former theory, in that a

ribosomal protein, S6, has been shown to be phosphorylated *in vivo* in response to cAMP.

Cyclic AMP has been shown not only to transcriptionally activate individual eukaryotic genes (see above), but also to activate "gene families." This rather interesting effect was noted by Huang et al. (1984), while investigating the protective effect of dbcAMP upon the development of mammary carcinomas in rats exposed to DMBA. DMBA is one of the most carcinogenic polycyclic hydrocarbons known, and is believed to bind to DNA and somehow initiate a transformation process. Pretreatment of rats with dbcAMP blocks DMBA-induced mammary tumor production, presumably at a transcriptional level. *In vitro* translation of poly A^+ RNA from young rat mammary glands treated with dbcAMP resulted in a spectrum of proteins resembling those seen in glands from old, untreated rats. This pattern of gene expression, characteristic of a fully differentiated, non-proliferating gland is resistant to the effects of DMBA. Huang et al. conclude by asserting that dbcAMP may exert its effect by imposing specific patterns of transcription that lead to the loss of growth potential, and a promotion of the differentiative process. Thus, here is restricted, yet definitive evidence of the action of dbcAMP upon a family of gene transcripts. It is an

effort to go slightly beyond a phenotypic description of cAMP-induced maturation, and attempts to describe the programmed differentiative pathway of a particular lineage.

There are conflicting reports regarding the role of cAMP in mediating the immune response. Webb et al (1977) used an in vivo system in which the injection of heterologous erythrocyte antigens (sRBCs) induced rapid, transient changes in splenic cAMP and prostaglandin levels. Using NZB mice, a lineage that is known to exhibit decreases in immunocompetence with age, the workers showed a correlation between immune function and cAMP responsiveness. They conclude that there is likely a defect in cyclic nucleotide or prostaglandin metabolism that is responsible for the altered state of immune reactivity. They fail to elucidate the level of this proposed defect, nor do they isolate the affected cell population, but they do establish a compelling contributory role of cAMP to the immune response.

More recent studies have focused upon the modulation of mitogenic signals in lymphocytes by cAMP. Most workers agree that the treatment of T lymphocytes with either cAMP analogs or agents known to increase intracellular cAMP, result in an inhibition of blastogenic responses to mitogens and antigens. Differential

inhibitory effects on lymphocyte proliferation induced by different mitogens have been seen with agents that increase cellular cAMP. Novogrodsky et al. (1983) conclude that in murine thymocytes, the inhibitory effect seen after exposure to cAMP-elevating agents is the result of interference with induced IL-2 synthesis, as opposed to a blockage in DNA synthetic machinery. This inhibition is dependent upon the stage of the thymocyte proliferative pathway at which the intracellular cAMP concentration is raised.

Exogenously added dibutyryl cAMP will effectively block the action of T cell mitogen-induced suppression of an *in vitro* immune response, by inhibiting the production of interferon by the T lymphocytes, and preventing the development of a suppressed state (Johnson, 1977). In addition, cAMP at levels of about 1 mM will inhibit interferon production in PHA-stimulated leukocytes, as well as in virus or polynucleotide stimulated L cells (Epstein and Bourne, 1976). Both of these laboratories invoke a cAMP-mediated gene regulatory mechanism, differing only in the affected gene.

The B lymphocyte system has also been examined in terms of cAMP effects. Ohara and Watanabe (1982) established a protocol for intracellular microinjection, using erythrocyte ghosts as a vehicle for transport.

They found that microinjection of anti-cAMP antibodies into murine splenic lymphocytes markedly enhanced the proliferative responses to mitogens, LPS, or Con A. Further, in cells containing these antibodies, the degree of cytoplasmic protein phosphorylation was markedly reduced. They conclude that there is a change in intracellular cAMP levels in lymphocytes at a very early stage of stimulation, which can act to modulate the rate of proliferation in those cells.

Burchiel et al. (1984) utilized cAMP production in cloned murine lymphoid tumor cell lines to study cell differentiation. Their approach was to study panels of T, B, and macrophage tumors representing various stages of differentiation, and assay for the presence of various cell surface receptors (such as prostaglandin, histamine, and cholera toxin). The functional assay relied upon increases in the level of intracellular cAMP following exposure to the appropriate pharmacological agent (presumably due to physiological receptor stimulation). This study marked a departure from typical immunological or labelled ligand analyses of cell surface markers. Because of the complex biochemical endpoint chosen (cAMP levels), however, it is difficult to equate the results with specific levels of pharmacological receptors present. In addition, the variances in the basal level

of adenylate cyclase activity in the various lines is not addressed. Despite these problems, the study has provided some interesting findings. The only B cell tumor that demonstrated responsiveness to pharmacologic agents was the WEHI-231 line, which also happened to represent the earliest stage of differentiation in the study. This study lends credence to the possibility that a "window" of sensitivity to cAMP-elevating agents exists during B cell differentiation, important in a hormone-dependent B cell differentiative process. These workers also demonstrated that murine T cell lines are more susceptible to a drug or hormone-induced cAMP elevation than B cell lines. This heightened sensitivity has been reported previously (Vischer, 1976), and may imply an increased drug or hormone receptor population on thymic-derived cells.

The described studies of cAMP effects on the immune system were done using both normal and tumor cells. Several workers have sought to correlate cAMP levels with cellular proliferation. Studies by Hait and Weiss (1976) have shown that the concentration of cAMP is lowest during the mitotic stage of the cell cycle, and is inversely related to the rate of cell growth. In contact inhibited cell populations, the cAMP level is elevated. A comparison of malignant versus normal cell analogues

revealed that cAMP is lower in cells transformed *in vitro* by oncogenic viruses and in certain in vivo tumors. Also, exogenously added cAMP (or the addition of agents that raise intracellular cAMP levels) was found to decrease the cellular growth rate, and to increase biochemical and morphological differentiation. In a comparison of transformed versus non-transformed lymphocytes from the same mouse strains, it was demonstrated that the observed cAMP-induced growth inhibition in leukemic lymphocytes was due to an increase in the activity of phosphodiesterase, the enzyme responsible for cAMP hydrolysis.

From this brief review, both the ubiquity of cAMP as a regulatory molecule in prokaryotes and eukaryotes, as well as the diversity in the range and functions of the gene products under its control have been explored. Future studies will no doubt uncover fascinating associations between cAMP and cellular regulation.

CHAPTER II

MATERIALS AND METHODS

Cells and Culture Conditions

The FLE1-4 cell line was derived from 15-day BALB/c fetal liver cells transformed by the P120 strain of Abelson murine leukemia virus (Siden et al., 1985). The RLØ$^{-}$ 11 cell line is a cloned subline derived from *in vitro* passage of RLØ$^{-}$1, a radiation-induced BALB/c thymoma, and was obtained from N. Rosenberg (Tufts University, Boston, MA). Mus F clone D (MFCD) is a line derived from an AKR mouse thymoma, and was obtained from D. Steffan (Worcester Foundation, Shrewsbury, MA). NALM-16 is a human acute lymphoblastic leukemia line obtained from J. Minowada (Roswell Park Memorial Institute, Buffalo, NY). All murine lymphoid lines were maintained in RPMI 1640 media (GIBCO, Grand Island, NY) supplemented with 10% heat-inactivated fetal calf serum (HyClone, Logan, UT), 50 uM 2-mercaptoethanol, 2 mM glutamine, and 50 ug/ml gentamicin sulfate (Valley Biologics, State College, PA). Cells were grown at 37^{o}C in a humidified atmosphere of 5% CO_2.

Enzymes and Chemicals

Restriction enzymes were purchased from New England Biolabs or Bethesda Research Laboratories. Digestions were carried out under the conditions suggested by the manufacturer, or in TA buffer (33 mM tris-acetate pH 7.9, 66 mM potassium acetate, 10 mM magnesium acetate, 0.5 mM dithiothreitol, and 100 ug/ml nuclease-free BSA) as described by O'Farrell (1980). Lysozyme was puchased from Miles Laboratories and was stored at -20°C. Deoxyribonuclease I (DNAse I) was purchased from P-L Biochemicals. For RNA isolations, the DNAse was rendered RNAse-free by incubation at 1 mg/ml in 20 mM Tris-hydrochloride pH 7.4, 10 mM $CaCl_2$ at 37°C for 20 min, as described by Tullis and Rubin (1980). Proteinase K was then added in the same buffer to a final concentration of 50 ug/ml and the incubation continued for another 2 hrs. Proteinase K was purchased from Sigma Chemical Co., and was stored desiccated at -20°C. All phenol was from Fisher Co., and was redistilled prior to use. Chloroform used throughout this work refers to a mixture of 96% chloroform and 4% isoamyl alcohol. Prostaglandin E1, deoxybromouridine (BUdR), cis-retinoic acid, and aphidicolin were obtained from Sigma Chemical Co. Interleukin 2 (IL-2), in the form of concanavalin A-

stimulated EL-4 cell-free supernatant was the kind gift of R.T. Smith (University of Florida). Interleukin 3 (IL-3), in the form of WEHI-3 cell supernatants, was supplied by S. Russell (University of Florida). Parabactin was purified and provided by R. Bergeron (University of Florida).

Nucleic Acid Probes

Genomic and cDNA murine terminal deoxynucleotidyl transferase clones were the generous gifts of N. Landau and D. Baltimore (Whitehead Institute, Cambridge, MA) (Landau et al., 1985). A murine genomic EcoRI clone (pR1-C_u) containing the immunoglobulin mu constant region (Alt et al., 1981) was obtained from F. Alt (Columbia University, New York, N.Y.). An Abelson murine leukemia virus subclone, pAB3Sub3, was obtained from S. Goff (Columbia University, New York, N.Y.).

Plasmid DNA Preparation

To obtain preparative amounts of plasmid DNAs, plasmid-containing Escherichia coli HB101 or MC1061 was grown in Luria (L) broth (1.0% tryptone, 0.5% yeast extract, 0.5% NaCl, 0.5% glucose, and appropriate

antibiotic (tetracycline 20 mg/l; ampicillin 100 mg/l) (final pH 7.4)) in a shaker-incubator (New Brunswick Scientific Company) at 37°C until a Klett value of 80 was achieved, as measured with a green filter (Klett-Summerson colorimeter). The plasmids were then amplified by the addition of chloramphenicol to 170 ug/ml and continued incubation for 16-20 hrs. Plasmid isolation was accomplished using a modification of the technique described by Maniatis et al. (1982). The cells were pelleted by low speed centrifugation and the pellet was washed once with 50 mM Tris-hydrochloride (pH 7.5). The washed cells were resuspended (in 10 ml/l culture) in 50 mM Tris-hydrochloride (pH 8.0), 25% sucrose, and placed on ice. Freshly prepared lysozyme (10 mg/ml stock in 0.25 M Tris-hydrochloride pH 8.0) was then added to a concentration of 1 mg/ml and the cells were incubated at 4°C for 5 min. Four milliliters of 0.2M EDTA (pH 8.0) were added (per liter of initial culture), and the sample was gently mixed and incubated at 4°C for 5 min. Detergent mix, consisting of 62.5 mM EDTA, 50 mM Tris-hydrochloride (pH 8.0), 1% Nonidet P-40 (Sigma), and 4 mg/ml sodium deoxycholate (DOC) was added at a volume of 16 ml/l initial culture, and the sample was incubated at 4°C for 10 min. The sample was then centrifuged at 24,000 rpm (Beckman SW 27 rotor) for 2 hrs and the

supernatant collected. Form I DNA was then purified by equilibrium centrifugation on a CsCl/ethidium bromide gradient (density = 1.56 g/ml). The form I band was collected from the gradient and the ethidium bromide was removed by repeated extraction with water-saturated n-butanol (usually 5-6 extractions). The sample was dialyzed against 10 mM Tris-hydrochloride (pH 7.5) to remove the cesium chloride. Finally, the DNA was precipitated with ethanol, dissolved in 10 mM Tris-hydrochloride (pH 7.5), 1 mM EDTA, and stored at $4^{o}C$.

RNA Isolation

Two methods of RNA isolation were regularly employed. All glassware was baked, and all solutions were autoclaved prior to use. DEPC treatment was not necessary, and contraindicated in procedures requiring translatable mRNA.

When poly (A) mRNA was sought, a modification of Wiegers method was used (Wiegers and Hilz, 1971). Washed cell pellets (either fresh or frozen) of up to 10^{9} cells (or up to 1 gm of tissue) were resuspended in 10 mls of extraction buffer containing: 0.1 M NaCl, 0.02 M Tris-hydrochloride (pH 7.4), 1 mM EDTA, 1.0% SDS, and 1 mg/ml freshly added Proteinase K. The cells were then

disrupted using 5-10 strokes in a Dounce homogenizer, and sonicated extensively. Usually, three 15 second bursts of sonication, interrupted by 15 second cooling periods on ice were sufficient to reduce the viscosity of the solution. The extract was then incubated at 37°C for 45 minutes. Proteins were removed by the following extraction procedure: 2 phenol extractions (redistilled phenol equilibrated with 50 mM Tris-hydrochloride, pH 7.5); 1-phenol:chloroform extraction (1:1 v/v); 2 Chloroform (with 4% Isoamyl alcohol) extractions. Sodium Acetate (pH 5.5) was added to the aqueous phase to a final concentration of 0.3 M, and 2.5 volumes of ethanol were added. The solution was precipitated for at least 12 hours at -20°C. This method has been shown to give the best yield of RNA. There is considerable DNA contamination (small fragments due to sonication), so oligo dT selection must be performed prior to use.

When total RNA was desired, a procedure outlined by Maniatis was used (1982). Briefly, washed cell pellets of up to 10^8 cells were resuspended in 3.4 ml of an extraction buffer composed of 6 M guanidine isothiocyanate (Kodak), 5 mM sodium citrate pH 7.0, 0.1 M 2-mercaptoethanol, and 0.5% Sarkosyl. The samples were then sonicated twice, for 15 seconds (Branson Instruments), overlayered onto a 1.2 ml cushion of 5.7 M CsCl

(in 0.1 M EDTA) in SW 50.1 nitrocellulose centrifuge tubes (Beckman), and centrifuged at 35,000 rpm for 12-16 hrs at 15°C. The pellets were dried under vacuum, resuspended in 500 ul of 10 mM Tris-hydrochloride (pH 7.4), 1 mM EDTA, and extracted with an equal volume of a 4:1 mixture of chloroform and water-saturated n-butanol. The samples were adjusted to 0.3 M sodium acetate (pH 5.5) and precipitated with 2.5 volumes 95% ethanol.

Polyadenylated mRNA Isolation

Polyadenylated mRNA was isolated by chromatography over oligo(dT)-cellulose, as described by Aviv and Leder (1972). Total RNA (or total nucleic acid) was suspended in autoclaved H_2O at a concentration of approximately 5 mg/ml, denatured at 68°C for ten minutes, and quick-cooled on ice. An equal volume of 2x loading buffer was then added to the RNA, and the solution was allowed to attain room temperature prior to loading. Loading buffer (1x) is composed of: 20 mM Tris-hydrochloride (pH 7.5), 1 mM EDTA, 0.5 M NaCl, and 0.5% SDS. A column was constructed from a one milliliter syringe barrel, and loaded with 0.5 ml packed bed volume of type III oligo(dT) cellulose (Collaborative Research) in 1x loading buffer. Samples were passed through the column

three times, at a constant, gravity controlled flow rate of about 0.5 ml/min. Non-adenylated nucleic acid was washed off the column with wash buffer (10 mM Tris-hydrochloride (pH 7.5) and 0.5 M NaCl) until both SDS and nucleic acid stopped eluting (at least 10 column volumes). Poly A RNA was eluted in autoclaved water in 0.4 ml aliquots.

Fractions containing RNA were identified by spotting 2 ul onto 0.8% agarose plates containing 0.5 ug/ml ethidium bromide and visualizing under UV light. Carrier RNA (yeast) was usually added to the pooled fractions, which were then precipitated in sodium acetate (C_f=0.3 M) with 2.5 volumes of 95% ethanol.

Glyoxal RNA Denaturation and Electrophoresis

Ribonucleic acid (RNA) samples were denatured using glyoxal, as described by McMaster and Carmichael (1977). Appropriate quantities of RNA, in volumes of 11 ul or less, were combined with 4.8 ul of 10x PE buffer (1x PE= 10 mM $NaPO_4$ (pH 6.8), 1 mM EDTA). Eight microliters of 6M glyoxal (Sigma Chemical Co.) were then added, along with 24 ul of spectrophotometric grade dimethyl sulfoxide (DMSO) (Aldrich Chemical Co.). Autoclaved water was added to bring the final volume up to 48 ul. The samples

were then mixed well, and heated to 50°C for 60 min, then cooled on ice. Ten microliters of loading buffer (50% glycerol, 0.1% bromphenol blue) were added to each, and samples were either loaded immediately onto a gel or frozen (-20°C) until use.

Neutral 1% agarose gels, prepared in 1x PE were normally utilized. The tray buffer was also 1x PE. Gels were routinely electrophoresed for 1.5-2 hrs at 100 volts constant voltage with no recirculation. The bromphenol blue dye front migration was between eight and ten centimeters from the wells. This method allowed for complete denaturation of the sample as determined by previous experimentation.

Northern Blotting and Hybridization

Glyoxalated, electrophoresed RNA was transferred to nylon filters (Pall Co.) using a standard capillary transfer technique (Southern, 1975) in 20X SSC (SSC= 0.015 M sodium citrate, 0.15 M NaCl, pH 7.4). Transfers were allowed to proceed for at least 12 hrs, after which time the filters were heated for 1 hr in an 80°C vacuum oven. This procedure not only fixed the nucleic acid to the filter, but also inactivated the glyoxal. The

filters were stored at room temperature until used for hybridization.

Filters were prehybridized and hybridized as per manufacturer's specifications (Pall Co.). The nylon filters were prehybridized in 10-20 mls of a solution containing 50% formamide (Fisher Co., reagent grade), 5X SSC, 5X Denhardt's solution (1X= 0.02% Ficoll, 0.02% polyvinylpyrrolidone, and 0.02% bovine serum albumin), 0.1% SDS, 50 mM $NaPO_4$ (pH=7.4), and 250 ug/ml denatured salmon sperm DNA (Sigma Co.). The filters were heat sealed in boil bags (Sears) and incubated at 42^oC with shaking for 4-12 hrs. The prehybridization solution was removed and the filters were hybridized for 16-24 hrs at 42^oC in 15-20 ml of a solution which contained 50% formamide, 5 x SSC, 5 X Denhardt's solution, 0.1 % SDS, 50 mM $NaPO_4$ (pH 7.4), 0.1 mg/ml denatured salmon sperm DNA, and 1-2.5 x 10^6 cpm/ml denatured ^{32}P-DNA. Following hybridization, the filters were washed four times (5 min each) in 2 X SSC, 0.1% SDS, and then at 50^oC twice (15 min each) in 0.1 X SSC, 0.1 % SDS. The filters were then air-dried and autoradiographed at -70^oC with XAR-5 X-ray film (Kodak) and a DuPont Cronex intensifying screen.

Nick Translation

Cloned DNA was radiolabelled with ^{32}P for use as probe using the in vitro nick-translation procedure (Rigby et al., 1977). The reaction mixture (30 ul) contained: 0.25 ug DNA, 0.1 mM each of dGTP,dATP, and dTTP, 0.1 ng of freshly activated DNase, 50 mM Tris-hydrochloride (pH 7.9), 5 mM $MgCl_2$, 1 mM dithiothreitol, 50 ug/ml BSA, 100 uCi of α-^{32}P-dCTP (specific activity > 400 Ci/mmol, Amersham Corporation), and 2 units of DNA polymerase I (Boehringer-Mannheim). The DNAse had been activated prior to use by incubation at $0^{o}C$ for 2.5 hrs in an activation buffer (10 mM Tris-hydrochloride (pH 7.5), 5 mM $MgCl_2$, 1 mg/ml BSA, and 0.1 ug/ml DNAse). The labelling reaction was incubated at $14^{o}C$ for 30-45 min, and terminated by the addition of EDTA to a final concentration of 50 mM. Unincorporated deoxynucleoside triphosphates were then separated from labelled DNA by chromatography over Sephadex G-100 (Pharmacia Fine Chemicals) in 10 mM Tris-hydrochloride (pH 7.5), 1 mM EDTA. Fractions which contained the void volume were pooled and stored at $4^{o}C$ until used. The specific activity was routinely greater than 1×10^{8} dpm/ug DNA.

In Vivo Labelling and Immunoprecipitation

Cells for in vivo labelling were harvested, washed twice in balanced saline solution (BSS), and resuspended at 1 x 10^7 cells/ml in MEM minus methionine (GIBCO Laboratories). Twenty-five to 50 uCi/ml of ^{35}S-methionine (1200-1400 Ci/mmol, Amersham) were added, and the cells were incubated for 1-2 hrs at 37^{o}C, harvested, and either used immediately for immunoprecipitation or frozen as pellets at -70^{o}C.

Labelled cell pellets were suspended at 2 x 10^6/ml in ice cold phosphate-buffered detergent lysis buffer (PLB): 10 mM $NaPO_4$ (pH 7.5), 0.1 M NaCl, 1% Triton X-100 (Packard Instrument Co.), 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate (SDS). PMSF was immediately added to a final concentration of 1 mM, and the sample was vortexed and centrifuged at 2000 rpm for 10 min at 4^{o}C. The supernatant was collected and centrifuged at 45,000 rpm (Beckman 50Ti rotor) for 1 hr at 4^{o}C. A small sample (5 ul) of this cleared cell lysate was routinely precipitated in 5% TCA to determine labelling efficiency, and the remainder was used for immunoprecipitation.

Antisera were cleared by centrifugation for 10 min at 12,500 x g in an Eppendorf centrifuge at 4^{o}C, and

added to cleared lysates containing no more than 1×10^7 cpms as determined by TCA precipitation. Appropriate volumes of antisera had been empirically determined previously, and varied for each antiserum used. The samples were mixed well, and incubated for 12-16 hrs at $0^{o}C$. Duplicate immunoprecipitations with normal sera were routinely performed.

Protein A-bearing Staphylococcus Aureus (Staph A) was grown and formalin fixed as described by Kessler (1975). The Staph A cells were incubated overnight at $4^{o}C$ in PLB containing 10 mg/ml BSA prior to use.

Fifty microliters of a 10% suspension of the washed staph cells were added to each immunoprecipitate, and incubation was continued at $0^{o}C$ for an additional 2 hrs. The protein A-antibody precipitates were collected by centrifugation (12,500 x g, 30 seconds), washed twice in PLB, and resuspended in 50 ul of protein sample buffer containing: 0.0625 M Tris-hydrochloride pH 6.8, 2% SDS, 10% glycerol, 70 mM 2-mercaptoethanol, and 1 mg/ml Bromphenol blue (Laemmli, 1970). Samples were then incubated at $68^{o}C$ for 30 min, centrifuged for 3 min, and supernatants were either electrophoresed immediately or frozen at $-20^{o}C$.

SDS-Polyacrylamide Gel Electrophoresis

Immunoprecipitated proteins were fractionated by discontinuous SDS-polyacrylamide gel electrophoresis as described by Laemmli (1970). Electrophoresis was at 150 volts through a 5% stacking gel and an 11.25% resolving gel (15 x 19 x 0.15 cm) until the bromphenol dye was near the bottom of the gel. The proteins were then fixed and stained in the gel by immersing the gel in a solution containing 45% methanol, 10% glacial acetic acid, and 0.25% Coomassie Brilliant Blue R for 15 min, then destained in excess 10% glacial acetic acid for 12-16 hrs. The gel was then washed extensively (30 min) in distilled water, and fluorographed by immersion into 1 M sodium salicylate for 40 min. Prior to autoradiography, the gel was dried by the use of heat and vacuum. The marker proteins used were either ^{14}C labelled (BRL) or unlabelled (Sigma) commercial preparations, and included myoglobin, phosphorylase B, bovine serum albumin, catalase, and cytochrome c.

Antisera

Rabbit anti-calf terminal deoxynucleotidyl transferase sera were obtained from A. Silverstone (Memorial

Sloan-Kettering Cancer Center, New York, NY). The polyclonal antisera were obtained from rabbits that had been immunized with alum purified calf terminal deoxynucleotidyl transferase (Silverstone et al., 1980).

Normal rabbit sera used in preliminary experiments were the pre-immunization sera from the same rabbits used above. Normal rabbit sera used in subsequent experiments were the kind gift of M. Labow.

Terminal Deoxynucleotidyl Transferase Activity Assays

Terminal deoxynucleotidyl transferase was assayed essentially as described by Coleman (1977). Cells were harvested, washed once in BSS, and resuspended at 1 x 10^8 cells/ml in 0.25 M KPO_4, 1 mM 2-mercaptoethanol. Cells were then sonicated twice for 15 sec, with a 15 sec refractory period on ice in between. Previous experimentation had determined that insufficient sonication resulted in low activity, while extensive sonication sheared chromosomal DNA and created numerous endogenous primers. Lysates were then centrifuged at 2000 rpm for 10 min at 4^oC and the supernatants were further clarified by centrifugation for 2 hrs at 45,000 rpm (Beckman 50Ti rotor) at 4^oC. Dialysis, when performed, was against 0.25 M KPO_4, 1 mM 2-mercaptoethanol for 12 hrs at 4^oC.

Reactions (50 ul) were carried out in buffer containing 0.2 M potassium cacodylate (pH 7.5), 1 mM $MnCl_2$, 1 mM 2-mercaptoethanol, 20 ug/ml $d(pA)_{12-18}$, 0.025 mM dGTP, 5 pmol α-^{32}P-dGTP, and 30 ul of cleared cell lysate (concentration 10^8 cells/ml), and incubated at 37°C for 30 min. Reactions without the d(pA) primer, or containing 1 mM ATP (Tdt specific inhibitor) were routinely performed as controls, to confirm primer dependence of the reaction and to ensure that the product was the result of Tdt polymerization, respectively. Incorporation was monitored either by TCA precipitation, spotting on DEAE-81 (Whatman Co.) paper and batch washing in several changes of 5% KPO_4 buffer, or by descending chromatography on DEAE-81 paper in a 5% KPO_4 buffer. Product was retained at the origin, while unincorporated nucleotides migrated with the solvent front. Spots were visualized by autoradiography, or counted in a liquid scintillation counter.

RNA Sucrose Gradient Size Fractionation

Polyadenylated mRNAs from RL cells were fractionated in a neutral sucrose gradient, using a modification of a procedure described by Haseltine (1976). Polyadenylated RL RNA (up to 150 ug/13 ml gradient) was precipitated in

ethanol and resuspended in 99% DMSO (Sigma Co.), 10 mM Tris-hydrochloride pH 7.5 at a concentration of 0.5 mg/ml. The samples were then heated at 37°C for 5 min, diluted four-fold in 5 mM Tris-hydrochloride pH 7.5, 1 mM EDTA, 0.5% SDS, heated for 2 min at 68°C, and cooled on ice prior to loading on gradients. Thirteen ml, 15-30% sucrose (RNAse-free, Schwartz-Mann Co.) gradients were prepared in 0.1 M NaCl, 1 mM EDTA, 5 mM Tris-hydrochloride pH 7.5, 0.5% SDS, loaded in SW 41 tubes (Beckman), and allowed to settle for 1 hr at room temperature. Gradients were centrifuged at 35,000 rpm for 6 hrs at room temperature (22°C). Fractions (400 ul) were collected by puncturing the bottom of the tubes. Marker RNA (E. coli rRNA) was routinely centrifuged opposite the RL RNA and was fractionated on a similar gradient. Ten micrograms of proteinase K-treated, phenol extracted glycogen (type VII from oysters, Sigma Chemical Co.) was added to each fraction as carrier, and samples were ethanol precipitated for 16 hrs at -20°C. Migration of the marker rRNA samples was determined by gel electrophoresis. The rRNA gradient fractions were ethanol precipitated, resuspended in water, adjusted to 5 mM sodium borate, 10 mM sodium sulfate, 1 mM EDTA (final buffer pH 8.2), 15 mM methyl mercury hydroxide and electrophoresed on a 1% agarose gel containing 5 mM

methyl mercury hydroxide (Alfa Products), for 2.5 hrs at 65 milliamps constant current (Bailey and Davidson, 1976). The gel was stained for 15 min in 2 ug/ml ethidium bromide and visualized under UV light (Fotodyne Transilluminator).

In Vitro Translation in Rabbit Reticulocytes

In vitro translation of fractionated mRNA was carried out as described (Pelham and Jackson, 1976). Young New Zealand White rabbits weighing 4-6 lbs were made anemic by subcutaneous injection of a 1.2% solution of acetylphenylhydrazine (Sigma) neutralized to pH 7.0 with 1 M Hepes (pH 7.5), according to the following schedule: 2 ml on day 1, 1.6 ml on day 2, 1.2 ml on day 3, 1.6 ml on day 4, and 2 ml on day 5. The rabbits were sacrificed on day 9, and blood was collected by cardiac puncture in 10 ml of chilled saline containing 0.001% heparin (Sigma). The blood was filtered through cheese-cloth and washed by centrifugation three times in chilled PBS. Packed cells were lysed at $0^{o}C$ for 1 min with an equal volume of cold distilled water containing 40 ug/ml hemin (Kodak). After 1 min, the lysate was centrifuged at 15,000 rpm for 20 min at $4^{o}C$. Aliquots of 200 ul were

immediately frozen in liquid nitrogen, where they were stored until use.

Endogenous reticulocyte RNAs in the lysate were destroyed by treatment with micrococcal nuclease prior to use. To one milliliter of thawed (on ice) lysate was added 10 ul of hemin stock solution (20 mg hemin, 0.4 ml 0.2 M KOH, 0.6 ml sterile water, 0.1 ml 1 M Tris-hydrochloride pH 7.8, 4 ml ethylene glycol), 20 ul 50 mM $CaCl_2$, and 0.5 ul micrococcal nuclease (150,000 units/ml in 50 mM glycine pH 9.2, 5 mM $CaCl_2$, BRL). The mixture was incubated for 15 min at 20^{o}C, and terminated by the addition of 20 ul of 0.1M EGTA pH 7.0 and 0.4 ul creatine phosphokinase (40 mg/ml in 50% glycerol/water; Sigma, type I). The lysate was then aliquotted, flash-frozen in liquid nitrogen, and stored there until used.

Translation reactions (25 ul) were composed of the following: 0.5 mM spermidine, 8 mM creatine phosphate, 0.02 mM amino acid mix (-cys, -met), 1.6 mM dithiothreitol, 20 mM Hepes pH 7.6, 0.08 M potassium acetate, 0.65 mM magnesium acetate, 15-25 uCi each ^{35}S-methionine and ^{35}S-cysteine (New England Nuclear), 10 ul nuclease-treated reticulocyte lysate, and up to 5 ug of oligo(dT) selected mRNA. The amino acid mix consisted of equimolar concentrations of eighteen standard amino acids, made up in sterile water. The reactions were incubated at 20^{o}C

for 60 min, and either prepared for immunoprecipitation immediately, or frozen at -20°C until use. Incorporation was monitored by TCA precipitation.

Induction of Terminal Deoxynucleotidyl Transferase

Induction of Tdt in FLE1-4 cells was accomplished as previously described (Siden et al. 1985). Cells were grown to high density (1 x 10^6/ml), pelleted by centrifugation, and resuspended in fresh medium containing 50 ug/ml each of caffeine and dibutyryl cAMP (Sigma) at a concentration of 8 x 10^5 cells/ml.

In Vitro Nuclear Transcription

Nuclear isolation and *in vitro* transcription reactions were carried out as described by E. Mather (La Jolla Cancer Research Foundation). Cells (1 x 10^8 minimum) were harvested and washed once in ice-cold BSS. Pellets were then resuspended in 10 mls of SMH buffer (0.32 M sucrose, 3 mM $MgCl_2$, 1 mM Hepes pH 7.5). Ten milliliters of SMHT buffer were then added (SMH as above with 0.1% Triton X-100 (Sigma) added), and the cells were homogenized with 6 strokes of a tight (type B) Dounce homogenizer. Aliquots were occasionally examined

microscopically with trypan blue dye to determine lysis. The sucrose concentration was then adjusted to 0.28 M with cold H_2O, and the sample underlayered with 10 ml SMH buffer. The sample was then centrifuged at 2000 rpm for 10 min at 4^oC, and the pelleted nuclei were resuspended by vortexing in 20 mls of SMH buffer. An aliquot was removed for counting, and the remainder of the sample was centrifuged as above. Nuclei were finally resuspended in GMET buffer (40% glycerol, 5 mM $MgCl_2$, 50 mM Tris-hydrochloride pH 8.0, 0.1 mM EDTA) at 2 x 10^8 nuclei/ml, and either used immediately, or frozen quickly on dry ice and stored at -70^oC until use.

Transcription reactions were performed in 200 ul volumes containing: 2 x 10^7 nuclei, 70 mM KCl, 10 mM $MgCl_2$, 1 mM dithiothreitol (freshly prepared), 1 mM each ATP, GTP, and CTP, 200-500 uCi α-^{32}P-UTP (approx. 600 Ci/mmol, New England Nuclear) plus unlabelled UTP to give a final concentration of 4 uM, 10 mM creatine phosphate, and 20 units/ml creatine phosphokinase (BRL). Reactions were incubated at 37^oC for 40 min, and frozen on dry ice and stored at -70^oC until processed.

Labelled RNA was isolated from nuclei using a procedure described by M. Labow (Princeton University). Thawed nuclear reactions (200 ul) were dissolved in 1 ml HSB (10 mM Tris-hydrochloride pH 7.4, 0.5 M NaCl, 50 mM

$MgCl_2$, 2 mM $CaCl_2$). One hundred microliters of 1 mg/ml RNAse-free DNAse were added and allowed to react at room temperature for 2 min, after which 4 mls of ETS (10 mM EDTA, 10 mM Tris-hydrochloride pH 7.4, 0.2% SDS) were added. An additional 200 ul of 0.5 M EDTA were added to each sample, followed by 4 mls of distilled phenol (equilibrated with 50 mM Tris-hydrochloride pH 7.0). The samples were incubated at 68°C for 15 min with occasional shaking. The samples were then quick-cooled on ice, and the aqueous layer extracted. Following a phenol:chloroform extraction (1:1 v/v) and a chloroform extraction, 100 ug of yeast carrier RNA were added to each sample. Ethanol precipitation at -20°C for 12-16 hrs was performed using 0.3 M sodium acetate (pH 5.5).

Cloning of Non-Repetitive Tdt Intron Probe

The cloning of an appropriate, non-repetitive fragment of the genomic Tdt probe (see Chapter III) was undertaken to facilitate *in vitro* nuclear transcription anaysis of the expressed Tdt gene. Following isolation of the appropriate fragment by preparative electrophoresis, electroelution was used to extract the DNA from the gel matrix. After ethanol precipitation, the fragment was treated with Klenow fragment for 30 minutes as

described (Maniatis et al., 1982). The reaction was terminated by the addition of EDTA to 1 mM, and the DNA solution was adjusted to yield 20 mM Tris-hydrochloride, 0.2 mM NaCl. The DNA was then bound to and eluted from a commercially obtained matrix (Elu-Tip, Schleicher and Schuell, Inc.). Yeast tRNA was added as a carrier, and the blunt-ended insert fragment was ethanol precipitated.

Vector DNA (pUC-8) was the kind gift of G. Zambetti (University of Florida). Two and one-half micrograms of the DNA (in 5 ul of 10 mM Tris-hydrochloride pH 8.0) were suspended in 43 ul of a buffer containing: 50 mM Tris-hydrochloride (pH 8.0), 1 mM $MgCl_2$, 0.1 mM $ZnCl_2$, and 1 mM spermidine. The vector was then treated with 0.5 ul (0.12 U) calf intestinal phosphatase (CIP, Boehringer Mannheim) for 15 min at 37°C, followed by another 15 min incubation at 56°C. An additional 0.5 ul of CIP was then added, and the two-stage incubation procedure was repeated. The enzyme was then inactivated by the addition of 50 ul of a buffer containing 20 mM Tris-hydrochloride (pH 8.0), 0.2 M NaCl, 2 mM EDTA, and 1% SDS, and heating to 68°C for fifteen minutes. The DNA was extracted several times with phenol:chloroform (1:1, vol:vol), ethanol precipitated (with 0.3 M NaOAc), and resuspended in 5 ul of TE buffer (10 mM Tris-hydrochloride pH 7.6 and 1 mM EDTA).

Ligation was performed by mixing equimolar amounts of vector and insert yielding a solution of 1-2 ug total DNA in 20 ul ligation buffer (50 mM Tris-hydrochloride (pH 7.6), 10 mM $MgCl_2$, 20 mM dithiothreitol (freshly prepared), 1 mM ATP and 50 ug/ml BSA). The reaction proceeded at $4^{o}C$ for 16 hrs, with 200 U of ligase (New England Nuclear Co.) added both initially and at 4 hrs into the reaction.

Competent cells (E. coli strain MC1061, from E. Wakeland, University of Florida) were prepared as follows. One hundred milliliters of log phase MC1061 cells (OD_{590} = 0.375) were chilled on ice for 10 min, split into two aliquots, centrifuged at 3000 rpm in an IEC centrifuge for 5 min (at $4^{o}C$), and each pellet was gently resuspended in 10 mls of a $CaCl_2$ buffer (60 mM $CaCl_2$, 10 mM PIPES (pH 7.0), and 15% glycerol). The cells were then centrifuged again as above, and each pellet was again resuspended in 10 mls of $CaCl_2$ buffer, and incubated at $0^{o}C$ for 30 min. Cell were again centrifuged as described, and each pellet was resuspended in 2 ml $CaCl_2$ buffer, divided in 200 ul aliquots, and stored at $-80^{o}C$ until use.

The transformation reaction consisted of combining the DNA (in a 10 ul volume) with 100 ul of competent cells which had been thawed on ice. The mixture was

incubated at 37°C for 5 min, combined with 0.9 ml of prewarmed L broth, and incubated for 60 min at 37°C. Fifty to 100 ul of the transformation mixture were then spread on prewarmed L-amp plates, and incubated overnight at 37°C.

Cyclic AMP Assays

Intracellular cyclic AMP determinations were performed with a commercially prepareed assay system (Amersham Co.) using the recommended protocol. Briefly, cells were rapidly harvested and washed once in ice-cold BSS. Pellets were flash frozen by immersion in liquid nitrogen, and stored at -70°C until assayed.

The assay chosen was the non-acetylated cAMP radioimmunoassay, which is not as sensitive as the acetylated assay, but is more rapid and better suited to cell pellets containing variable levels of cAMP. Two concentrations of each sample were assayed in triplicate, and interpolated onto a standard curve, which was constructed using the supplied reagents. The graph of the standard curve obtained is found in Chapter III (Figure 3-29).

Flow Cytometric Analysis

Flow cytometry was performed using the methodology described by Bergeron et al. (1986). Briefly, aliquots of FLE cells which had been cultured in the presence of various inducing agents for varying periods of time were washed once in chilled phosphate buffered saline (PBS) and pellets were resuspended in 50% ethanol, stained with propidium iodide (Calbiochem Co.), and analyzed with a FACS II flow cytometer (Becton-Dickinson) interfaced with a microcomputer (Hewlett Packard 45B). DNA distributions were obtained from analyses of the red fluorescence from the propidium-stained DNA, and were determined with the kind assistance of Michael Ingeno (University of Florida).

Analysis of Histone Phosphorylation

Cells were labelled with inorganic phosphate, and histones isolated using a procedure from L. Green (University of Florida). Fifty milliliters each of 20 hr induced and uninduced FLE cells were centrifuged at 1500 rpm (IEC centrifuge), washed twice in Balanced Saline Solution (BSS), and resuspended in 50 mls of phosphate-free media (Gibco, Co.) plus or minus inducing agents,

plus 4 uCi of $H_3{}^{32}PO_4$ in H_2O (carrier free, 285 Ci/mg, ICN Biomedicals). The cells were incubated for 2.5 hrs at 37°C with gentle shaking, and washed three times in BSS, three times in 80/20 Triton (80 mM NaCl, 20 mM EDTA, 1% Triton X-100, pH 7.2), and twice in 0.5 M NaCl, 0.01 M Tris-hydrochloride pH 8.0. The nuclear pellets were each resuspended in 3.5 ml H_2O and kept on ice for 30 min. Chromatin was pelleted by centrifugation at 8000 rpm (Sorvall centrifuge) for 15 min (at 4°C). The remaining pellets were mixed with cold 0.4 M H_2SO_4 (500 ul) in an Eppendorf centrifuge tube, and kept on ice for 20 min. The pellets were extracted by centrifugation (10 min, 12500 x g, 4°C) and extracted proteins were precipitated by the addition of 2 vol 95% ethanol and overnight storage at -20°C. Following centrifugation (8000 x g, 4°C, 10 min), the pellets were resuspended in H_2O, and analyzed by PAGE as described above.

Unlabelled histone markers were either prepared as above from HeLa cells, or the kind gift of L. Green.

CHAPTER III

RESULTS AND CONCLUSIONS

Specificity of Antisera Used

The Tdt-specific antiserum used in this study has previously been shown to immunoprecipitate a 58 kD protein containing the predominant Tdt activity from murine thymocytes (Silverstone et al., 1980). Correlations between Tdt activity levels in several murine cell lines and absolute levels of immunoprecipitable protein were noted in the work of Siden and coworkers (1985), yet the exact relationship between these parameters was not quantitated. Since this antiserum was to be utilized throughout the course of the project in order to assay Tdt polypeptide in cell lysates, it was important to ensure its specificity by several criteria.

Figure 3-1 demonstrates an immunoprecipitation of metabolically radiolabelled proteins from dbcAMP-treated and untreated FLE murine pre-B cells, as well as RLσ^{+} 11 (abbreviated RL) transformed murine T cells, constitutive producers of Tdt (described below). Such an immunoprecipitation was routinely performed in all experiments

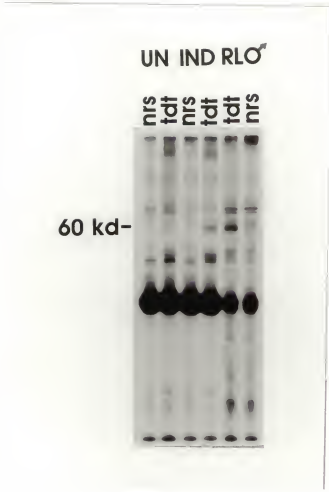


Figure 3-1. Immunoprecipitation of Tdt Protein.

Cells were induced as described in the text, biosynthetically labelled with a metabolic 2 hr ^{35}S-methionine pulse, and cleared cell lysates were immunoprecipitated with rabbit anti-calf Tdt (tdt) or normal rabbit serum (nrs). Immunoprecipitates were analyzed by 11.25% SDS-PAGE and autoradiography. UN and IND refer to uninduced and induced FLE1-4 cells respectively, and RLO$^{\sigma}$ is a constitutive Tdt-producing murine thymoma.

to ensure that enzyme induction had been achieved. The methodology employed is described in Chapter II. The molecular weight of the murine pre-B Tdt is approximately 60 kD based upon molecular weight standards. As seen in the figure, the quantity of Tdt markedly increases upon prolonged incubation with dibutyryl cAMP and caffeine. A slight yet detectable level of Tdt protein was occasionally observed in the uninduced FLE cell cultures. Non-specific background bands are readily identified by their presence in those samples immunoprecipitated with normal rabbit serum. No changes in the intensity of these background bands were observed following induction. There are several notable factors regulating the intensity of backgrounds seen in the immunoprecipitates.

Immunoprecipitations presented in this study occasionally revealed high levels of background signal using either normal rabbit serum or specific antiserum. Such backgrounds have been found to be the result of both individual differences between staph A preparations, and species-specific cross-reactivity. As seen in Figure 3-2, dual immunoprecipitations of the same metabolically radiolabelled cell lysates revealed stark differences when independent staph A preparations were utilized. In this case, aliquots from identical immunoprecipitated samples were treated with two different preparations of

Figure 3-2. Tdt Immunoprecipitation with Various Staph A Preparations

FLE cells were induced as described in the text, biosynthetically labelled with a metabolic 2 hr ^{35}S-methionine pulse, and cleared cell lysates were immunoprecipitated with rabbit anti-calf Tdt or normal rabbit serum using one of two independent Staph A protein preparations (Kessler, 1975). Immunoprecipitates were analyzed by 11.25% SDS-PAGE and autoradiography. Lanes are as follows: (a) and (c) are uninduced FLE cell lysates immunoprecipitated with nrs and anti-Tdt antisera, respectively; (b) and (d) are induced FLE cell lysates immunoprecipitated with nrs and anti-tdt antisera, respectively; (m) is a marker lane containing labelled RL cell lysate immunoprecipitated with anti-tdt antiserum and staph A protein from preparation number 1.

staph aureus (isolation described in chap II). Preparation 1 behaved typically, showing a slightly higher affinity for the antibody-antigen complexes than for the background proteins. Preparation 2 failed to bind the immunoprecipitated complex, instead binding background radiolabelled proteins non-specifically. Other staph preparations (not shown) exhibited an abnormally high level of background relative to the specific, F_c-mediated immune complex binding. No analysis was undertaken to ascertain the nature of these staph aureus differences. All were isolated from the same stocks, yet it is possible that following prolonged frozen storage, a proportion of the culture stock may have lost some F_c binding abilities. In an effort to reduce the incidence of non-specific protein binding, several measures were employed. These included the addition of excess cold (unlabelled) cell lysates from non-Tdt- producing cells (such as 2M3, an Abelson virus-transformed murine bone marrow line) which effectively served to compete with the labelled proteins without affecting the Tdt immunoprecipitation. Pre-incubation of the staph with this cold lysate prior to immune complex precipitation also was utilized successfully on occasion (data not shown). In addition, an effort was made to test several newly isolated strains of streptococcus that demonstrated F_c binding (kind gift of

M. Boyle). These strains, while effectively binding the immune complexes, bound non-specific proteins to a much higher degree than staph organisms (data not shown).

The species-specificity of the antiserum is also a contributory factor to the degree of background present in the immunoprecipitates. Figure 3-3 illustrates an immunoprecipitation of Tdt from a human leukemic cell lysate (NALM-16). A virtual absence of background was observed following normal autoradiographic exposures. A slight background could be seen after prolonged over-exposure, as in this figure. The antiserum used was raised in rabbits against bovine (calf) Tdt, and probably has a higher affinity for human Tdt than the murine enzyme (more immunological identity between human and bovine Tdt). The Tdt used as an immunogen was isolated and highly purified, so the likelihood of contaminants in the immunogen preparation was low. Background bands seen in murine samples probably reflect non-specific protein binding to the immunoadsorbant (staph). As long as the background bands in the immunoprecipitates proved non-specific (ie. were not increased in intensity in uninduced cell lysates), and did not interfere with the analysis of the specific Tdt immunoprecipitation, their presence was tolerated.

Figure 3-3. Immunoprecipitation of Tdt in NALM-16 Cells

NALM-16 human acute lymphoblastic leukemia cells were biosynthetically labelled with a metabolic 2 hr ^{35}S-methionine pulse, and cleared cell lysates were immunoprecipitated with normal rabbit serum (nrs) or rabbit anti-calf Tdt (tdt) serum as described. Immunoprecipitates were analyzed by 11.25% SDS-PAGE and autoradiography.

In order to further demonstrate the specificity of the antiserum used, Tdt activity assays were performed in the presence of either normal rabbit serum (pre-immunization serum) or Tdt antiserum. The inhibition curve seen upon addition of the Tdt antiserum is impressive (Figure 3-4). Terminal transferase activity was completely inhibited upon the addition of a volume of specific antiserum as low as 0.2 ul in a standard 50 ul reaction mixture. Normal rabbit serum failed to inhibit the reaction at volumes of up to 1 ul. Goat anti-mouse IgG antiserum and albumin failed to inhibit the Tdt activity assay, as well. Total protein determinations were performed upon both serum preparations, using the Bradford technique, and revealed nearly identical values (data not shown). In addition, the amount of staph A immunoprecipitable antibody (mainly IgG, which is the class expected upon repeated inoculation) present in both the normal serum and the antiserum preparations was determined by comparing densitometric tracings of Coomassie stained staph A precipitated immunoglobulin bands on polyacrylamide gels. Within the linear range of such an assay, the two sera again presented virtually identical profiles (data not shown). Thus, the inhibition of Tdt activity seen is probably due to specific

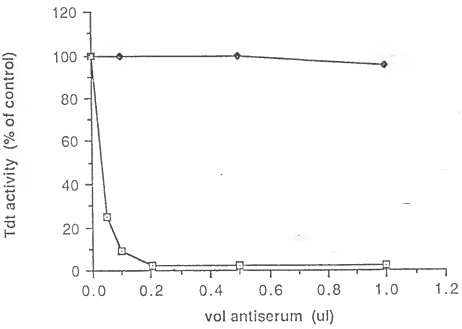


Figure 3-4. Inhibition of Tdt Activity from Induced Cells

Crude lysates (representing 3.0 x 10^6 cells) from induced cells were incubated for 2 hrs. at $0^{\circ}C$ in the presence of various volumes of either normal rabbit serum (solid circles) or rabbit anti-calf Tdt antiserum (open squares). Enzyme assays were performed as described. Standard deviations at each point were less than 10.8% of the plotted value, as determined by three replicates.

antibody-mediated inhibition, rather than non-specific protein effects.

Data obtained from immunoprecipitations of metabolically labelled cell lysates and Tdt activity assays have demonstrated the high specificity of the rabbit anti-calf Tdt serum used in the course of this project. The induction of Tdt and subsequent attempts to clone the Tdt gene were monitored using this antiserum in such assays.

Characterization of Tdt Activity in Induced and Uninduced FLE Cells

The immunoprecipitation data described above provided evidence of Tdt biosynthesis upon induction with dibutyryl cAMP and caffeine, yet revealed no information regarding the activity possessed by the protein. Activity analyses of cell lysates from induced and uninduced FLE cells and constitutive Tdt-producing RL cells were performed in order to determine whether Tdt biosynthesis parallels the enzymatic activity.

The cell line used throughout the course of these studies, FLE1-4, had been transformed, cloned, and characterized by E. Siden (1985) for susceptibility to Tdt induction by the exogenous addition of cyclic AMP. The experiments described herein utilized Tdt enzyme

induction as a requisite positive control, and the immunoprecipitation assay was extensively used as an indicator of successful induction (Figure 3-1). The presence of the biosynthetically labelled and immunoprecipitated Tdt band was associated with Tdt functional activity, in an assay described in Chapter II.

Figure 3-5 compares Tdt enzymatic activity in crude cell lysates from induced and uninduced FLE cells, and RL constitutive Tdt-producing cells. The activities are reported in terms of picomoles of dGTP incorporated onto oligo d(A) single-stranded primers in a thirty minute assay, and were calculated in terms of total protein concentration of the lysate (determined by Bradford analysis). There was approximately a two-fold difference between the Tdt activity in RL cells versus induced FLE cells. A low, albeit detectable level was usually seen in uninduced FLE cells. The activity assay was always performed in the presence and absence of primer, in order to exclude the possibility of endogenous DNA that might act as a primer and affect the results. Although the lysates were prepared by gentle sonication, there were occasional samples that contained excessive levels of fragmented genomic DNA which acts as a primer in the reaction. The primer dependency was previously shown to be directly related to the length of sonication during

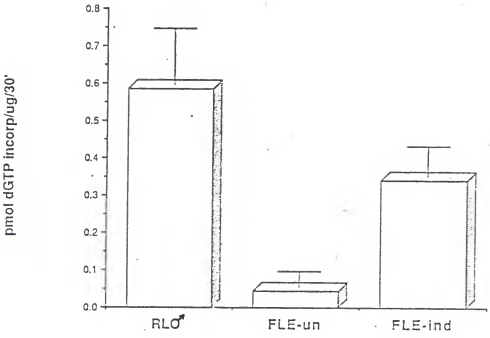


Figure 3-5. Enzymatic Assay of Tdt in Induced Cells

Crude lysates were prepared from induced and uninduced FLE1-4 cells and from RL♂ cells as described in the text, and assayed for terminal transferase activity (Coleman, 1977a). Individual backgrounds (reactions containing no exogenous primer) accounted for no more than 20% of total activity, and have been subtracted from each lysate assayed.

cell disruption (data not shown). Samples that demonstrated endogenous non-template directed synthesis at levels greater than 25% of total activity in the presence of exogenously added primer were not utilized.

The enzymatic activity profiles parallel the Tdt biosynthetic rates routinely observed in the immunoprecipitates (Siden et al., 1985). These data taken together might exclude the possibility that Tdt is regulated posttranslationally. That is, the correlation of specific protein with enzymatic activity lends support to the notion that newly translated Tdt possesses immediate activity. Indeed, biological precedent tends to enjoin a theory of cellular economy, which favors utilization of translated proteins. However, one should be mindful of an important caveat. As discussed previously (Chapter I), these samples are crude cell lysates, which are subsequently diluted and added to an _in vitro_ assay. So post-translational regulation, while unlikely, remains a possibility for Tdt regulation _in vivo_.

The kinetics of the Tdt activity from induced versus uninduced cell extracts was analyzed in order to ensure that the end-point anaysis (Figure 3-5) was consistent with an enzyme-mediated reaction (specifically with reported Tdt-mediated nucleotide addition curves), and that the uninduced level of Tdt was indeed a low basal

level throughout the course of the reaction. The analysis is illustrated in Figure 3-6. Crude lysates were assayed as above, with aliquots removed at various time points for analysis. The Tdt enzymatic reaction proceeds rapidly, with approximately 90% of nucleotide addition occurring within the first five minutes, under described reaction conditions. The methodology employed in these reactions was previously optimized to ensure adequate substrate saturation. The specific activity of the Tdt in induced and uninduced cells was not analyzed, owing to the difficulty involved in purifying the enzyme, but total protein determinations (Bradford) were performed on both induced and uninduced cell lysates, with only minor detectable differences (1.56 and 1.60 mg total protein/ml lysate, respectively).

Production of Terminal Deoxynucleotidyl Transferase Enriched cDNA

The initial strategy to ascertain the level of regulation of terminal deoxynucleotidyl transferase required the acquisition of a nucleic acid probe to analyze steady state levels and rates of mRNA synthesis for the gene. Because no probes were available when the project was initiated, a scheme for cloning the message sequences was devised. After consideration and testing

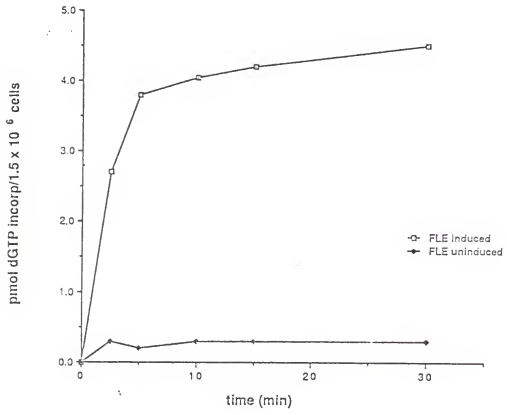


Figure 3-6. In Vitro Kinetics of Tdt Activity from Crude Cell Lysates

Crude lysates from induced and uninduced cells were assayed as described (Coleman, 1977a). At indicated times, aliquots were removed and specific incorporation was determined. Data represent the average of two experiments.

of various approaches to cloning, including polysomal immunoprecipitation of nascent Tdt peptide (with subsequent mRNA elution), and predictive DNA oligomer synthesis based upon a conserved region of the human and bovine Tdt amino acid sequence, it was decided to employ a method known as subtractive hybridization. Such a technique had been used successfully in the isolation of several genes, including dihydrofolate reductase (Alt et al., 1978), immunoglobulin J chain (Mather et al., 1981), and the T-cell receptor (Hedrick et al., 1984). Briefly, the technique involved hybridization of single-stranded cDNA from a Tdt-producing (induced) lymphoid cell line to mRNA from a non-producing (uninduced) lymphoid line. Those sequences that the two lines had in common (at equimolar concentrations) could be effectively removed by passage over hydroxylapatite, which binds double stranded nucleic acids. The unbound cDNA fraction would represent unique sequences, including Tdt. This cDNA would be cloned, and screened for Tdt-specific clones, using a hybrid-arrested Tdt translation assay. This assay depended upon successful <u>in vitro</u> translation of immunoprecipitable Tdt protein from mRNA from a constitutive Tdt-producing cell line. The RL murine thymoma line was selected as an appropriate constitutive Tdt producer after screening several available lines for

immunoprecipitable Tdt protein (Figure 3-7). In addition to possessing a high level of Tdt activity (Figure 3-5), the cells are well-suited to rapid, large scale growth (Silverstone et al., 1978). In vitro translation of Tdt had previously been reported using calf thymus RNA (Wolf et al., 1982), but never in the murine system. Early attempts to repeat the calf Tdt translations in our laboratory proved unsuccessful due to the degraded state of the calf thymus RNA isolated (data not shown). Even when isolated from fresh tissue that was immediately placed into a proteinase-containing buffer, the RNA was not completely intact or translatable.

A large-scale preparation of poly(A)-containing RL RNA was isolated and size fractionated by sucrose gradient centrifugation, as described in Chapter II. Samples were precipitated, washed, and alternating fractions were translated in a rabbit reticulocyte lysate containing ^{35}S-methionine. Translation products were then immunoprecipitated using the cross-reactive rabbit anti-calf Tdt antiserum (Silverstone et al., 1980), and analyzed by SDS-PAGE. Representative immunoprecipitates are shown in Figures 3-8 and 3-9 (a). Figure 3-8 depicts immunoprecipitated in vitro translation products obtained from alternate RNA gradient fractions. The background bands observed are non-specific, and are present in the

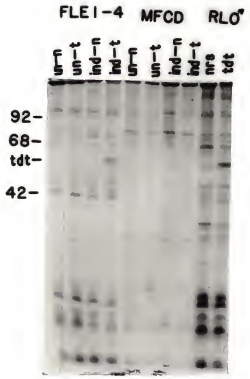


Figure 3-7. Tdt Immunoprecipitation in Various Cell Lines

Various cell lines were induced biosynthetically labelled with a metabolic 2 hr ^{35}S-methionine pulse, and cleared cell lysates were immunoprecipitated with normal rabbit serum (nrs) or rabbit anti-calf Tdt (tdt) serum as described. Immunoprecipitates were analyzed by 11.25% SDS-PAGE and autoradiography. The Mus F clone D (MCFD) line is derived from an AKR mouse thymoma.

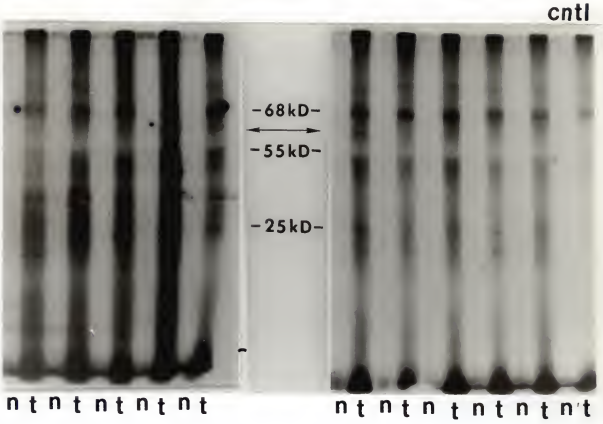


Figure 3-8. Immunoprecipitation of Tdt from RL Cell In Vitro Translation Products

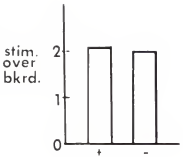
RL cell poly A^+ RNA was isolated and gradient purified as described. Alternating fractions were translated in a rabbit reticulocyte lysate in a reaction containing ^{35}S-methionine, and translation products were immunoprecipitated with normal rabbit serum (nrs) or rabbit anti-calf Tdt (tdt) serum as described. Immunoprecipitates were analyzed by 11.25% SDS-PAGE and autoradiography. The control lanes (cntl) represent immunoprecipitations of translation reactions containing no exogenous RNA.

immunoprecipitation of the uncharged reticulocyte lysate. The specific immunoprecipitated protein is approximately 58,000 daltons, which corresponds to both the reported molecular weight of murine Tdt (Silverstone et al., 1980) and the size of the in vivo metabolically labelled enzyme. Figure 3-9 (a) depicts the immunoprecipitated translation products from both a "Tdt-positive" and a "Tdt-negative" mRNA gradient fraction. The figure also includes a graph of ^{35}S-methionine incorporation in each of these fractions. This control is used to ensure that both fractions contain relatively equal amounts of translatable mRNA.

Terminal transferase activity was never detected in the in vitro translated products despite numerous attempts. Demonstrable Tdt activity from in vitro translated mRNA was not a prerequisite to cloning the gene, however, since the immunoprecipitation data (Figure 3-8) were acceptable. The requirements for expression and functional activity of an in vitro synthesized polypeptide are stringent, but obtaining such a result would have provided additional confirmatory evidence. The rabbit reticulocyte lysate in which the protein was synthesized routinely gave a high background when assayed by chromatography of crude cell lysates (Chapter II). Both dialysis and phosphocellulose microcapillary column

Figure 3-9. Preparation of Tdt-Enriched cDNA

(a) Poly (A^+), gradient-purified RNA fractions from RLO$^{\sigma}$ cells were translated in vitro in a rabbit reticulocyte lysate containing ^{35}S-methionine, immunoprecipitated with either normal rabbit serum (nrs) or rabbit anti-calf Tdt serum (tdt), and analyzed by SDS-PAGE (as described in text). Immunoprecipitations from representative Tdt-containing translation reactions (+) and Tdt-negative reactions (-) are shown. The accompanying graph illustrates the ^{35}S-methionine incorporation from each reaction, an indicator of translatable mRNA concentration; (b) The gradient-purified mRNA fractions used in (a) were subjected to Northern blot analysis using a radiolabelled Tdt-specific cDNA probe (Landau et al., 1985). Molecular weight determination is based upon migration patterns of murine rRNA species (28S = 5000 bases and 18S = 1950 bases; (c) Double-stranded cDNA prepared from mRNA fractions used in (b) were subjected to Southern blot analysis using a radiolabelled Tdt cDNA probe as above.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0
+
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IVT/IP
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+
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a

cDNA
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chromatography of translation products decreased the endogenous background, yet neither technique resulted in the recovery of detectable Tdt activity. Recovery of only unlabelled input oligo (dA) primer from the Tdt enzyme assays using oligo (dT) chromatography (and visualization on polyacrylamide-urea gels) supported the notion that the level of enzyme made in vitro was probably too low for this type of confirmatory analysis (data not shown). Control reactions performed with commercially prepared Tdt in the presence of reticulocyte lysate failed to demonstrate endogenous inhibitors of Tdt activity (data not shown). Microinjection and translation of the gradient purified mRNA in frog oocytes (kind gift of T. Hollinger) failed to yield measurable Tdt activity or even detectable immunoprecipitated Tdt.

A cDNA synthesis reaction was performed using both a "Tdt-positive" and a "Tdt-negative" fraction, based upon the immunoprecipitation of in vitro translation products, utilizing a technique described by Gubler and Hoffman (1983). Treatment of the cDNA reaction products with Klenow fragment of bacterial DNA polymerase I and T4 DNA ligase resulted in intact, blunt-ended molecules of up to 4 kB (as determined by denaturing gel analysis of radiolabelled reaction products, not shown). Tailing of the cDNA products synthesized (as well as restricted, blunt-

ended pBR322 vector) was achieved using commercially obtained terminal transferase, with approximately 15 d(C)'s added per vector end and 20 d(G)'s added per cDNA molecule terminus (the optimum for ligation, as determined by Peacock et al, 1979). Successful ligation was achieved (as monitored by electrophoretic mobility shift of radiolabelled insert fragments, not shown), and efforts were concentrated upon optimization of transformation.

At this stage of the project, the report of a murine Tdt cDNA clone came to our attention (Landau et al., 1985). Since the source of the clone was the RL line, the same one with which we were working, there seemed no point in continuing the cloning efforts. We obtained the cDNA clone (courtesy of N. Landau) and probed our presumptive Tdt positive and negative gradient-purified RNAs, as well as the cDNA that was synthesized from them. Figure 3-10 reveals successful gradient separation of Tdt mRNA, as evidenced by Northern blot analysis of purified fractions probed with the Tdt cDNA plasmid. The electrophoretic mobilities of HeLa 18S and 28S ribosomal RNAs (which had been centrifuged in a parallel gradient) suggest a mature Tdt mRNA size of approximately 2.1 kB (data not shown). The results seen in Figures 3-9 (b) and 3-9 (c) further demonstrate that an enrichment was

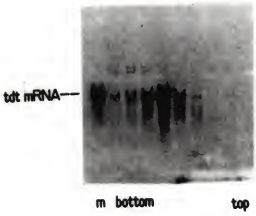


Figure 3-10. Detection of Tdt mRNA in RNA Gradient Fractions

RL cell poly A^+ RNA was isolated and gradient purified as described. Approximately 2 ug each of alternating RNA gradient fractions was electrophoresed through a 1% agarose gel, transferred onto a nylon membrane, hybridized to a radiolabelled Tdt cDNA probe, and analyzed by autoradiography. Lane (m) contains 5 ug of unfractionated RL cell poly A^+ RNA. Position of the fractions in the gradient are indicated by "top" and "bottom."

indeed obtained, and that the cDNA synthesis successfully yielded full length Tdt cDNA (approximately 2.1 kB; the same size as the mature Tdt mRNA). Thus it appears that our chosen methodology for cloning the Tdt gene was viable, and that the effort was progressing positively at the time of termination.

Steady-State Analysis of Tdt Specific mRNA

In order to begin to ascertain the level of molecular control of terminal transferase induction, Northern blot analyses of poly(A^+) mRNA were performed on induced and uninduced FLE1-4 cells. RL mRNA was included as a positive control. Figure 3-11 (a) shows an autoradiograph of mRNA from the three preparations hybridized to ^{32}P-labelled Tdt cDNA probe. The RL and the induced FLE cells have abundant Tdt mRNA, while the uninduced FLE cells do not. Since the mRNA preparations were quantitated by spectrophotometric analyses alone, a method subject to considerable variation, it was necessary to probe the same blot with a DNA species that was unaffected by the induction event. Because the cells were transformed with Abelson murine leukemia virus, a gene product previously determined to be independent of dbcAMP induction (E. Siden, personal communication), it seemed an appropriate

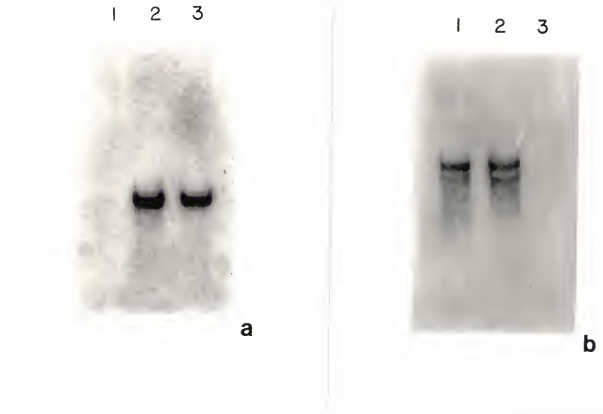
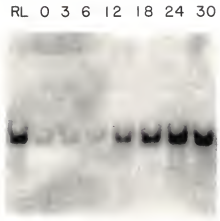


Figure 3-11. Induction of Tdt mRNA in FLE1-4 Cells

(a) FLE1-4 cells were induced as described. Poly (A^+) RNAs were denatured with glyoxal, electrophoresed through a 1% agarose gel, transferred to a nylon membrane filter, and hybridized to a radiolabelled Tdt specific probe. Lane 1, 10 ug uninduced FLE; Lane 2, 10 ug induced FLE; Lane 3, 4 ug RLO ; (b) Northern blot from (a) was stripped of Tdt probe and rehybridized to a radiolabelled Abelson murine leukemia virus specific probe (Goff et al., 1980).

control. Figure 3-11 (b) shows the same blot, stripped of the Tdt specific probe and rehybridized to a nick-translated clone containing a portion of the Abelson transforming gene. As expected, the Abelson transcripts were unaffected by induction, and the RL cell line, a radiation-induced thymoma, contained no v-Abl transcripts. This rehybridization was routinely done in all experiments necessitating quantitation of Tdt mRNA levels, in order to normalize RNA concentrations per lane accurately.

A kinetic analysis of steady-state Tdt gene expression following induction is shown in Figure 3-12. Poly-adenylated mRNA was obtained at various times post-induction, transferred to a nylon membrane filter, and hybridized to a nick-translated ^{32}P-labelled Tdt cDNA probe. As expected, the uninduced cells contained very little Tdt-specific mRNA. Terminal transferase message begins to accumulate at approximately six hours post-induction. This result correlates well with the appearance of Tdt immunoprecipitable protein and assayable Tdt activity in cell lysates (Siden et al, 1985). Figure 3-13 graphically illustrates the kinetics of Tdt mRNA accumulation. The units used are arbitrary, since they are based upon densitometric analysis of the autoradiographic Tdt band intensities relative to the



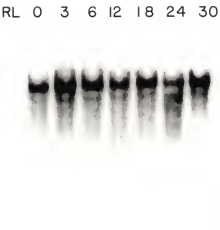


Figure 3-12. Tdt mRNA Accumulation During Induction

(a) FLE1-4 cells were induced as described at time 0. At various time points, 5 x 10^8 cells were removed, and poly (A^+) RNA was purified. Spectrophotometrically equivalent quantities (10 ug) of RNA from each time point were examined by Northern blot analysis, using a radiolabelled Tdt cDNA probe. Numbers refer to hours post-induction. RL0 Tdt marker lane contains 4 ug of poly (A^+) RNA; (b) Rehybridization of same blot (following Tdt probe removal) with Abelson murine leukemia specific probe.

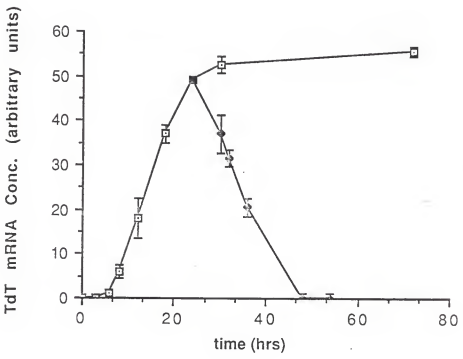


Figure 3-13. Kinetics of Tdt mRNA Accumulation

Northern blots representing seven time-course experiments (as in figure 3-12) were probed with a radiolabelled Tdt cDNA clone and band intensities were evaluated densitometrically. Open symbols represent Tdt mRNA content in FLE cells cultured for indicated time periods in the presence of dbcAMP and caffeine, while solid symbols represent Tdt mRNA content of the cells at indicated time points after removal of the inducing agents. Normalization of integrated intensity curves was achieved with the aid of a microcomputer, and values were expressed with respect to the 24 hr post-induction time point, as described in the text. Values for other time points on each blot were individually normalized to this band intensity, and the results were graphed.

background on the filter. Autoradiographs of Northern blots from seven Tdt induction time course experiments were scanned densitometrically. The areas under the curves were calculated (integrated) for each lane, from each experiment. The 24 hr time point was chosen as a reference, since it was included on each of the autoradiographs. The 24 hr time point from one experiment was chosen at random, and conversion factors were calculated to equate this lane to the 24 hr time point on all other blots. This method of standardization eliminated variability due to differences in probe specific activities and exposure times. The raw data from each gel were normalized, and the resulting graph is seen in Figure 3-13. Obviously, no standard deviation could be determined for the 24 hr samples, since they served as the reference points for each gel. Four of the radiographs evaluated included lanes of mRNA from cells in which the inducing agents were removed 24 hours post-induction, as seen in Figure 3-14. The curve generated following removal is shown in Figure 3-13. Upon returning to a basal level of Tdt expression, these previously induced cells can successfully be restimulated with dbcAMP and caffeine, and achieve similar maximum levels of Tdt mRNA (data not shown).

The induction curve of Tdt specific mRNA intensities with respect to time appears sigmoidal in shape. An equilibrium appears to be achieved between 24 and 30 hr post-induction. Steady-state Tdt mRNA accumulation has begun to plateau at this point, indicating that synthesis is maximal (or at least in equilibrium) with respect to message turn-over, which probably is maintained at a constant rate. Unless one invokes a specific Tdt mRNA degradation cascade which is responsive to varying Tdt mRNA levels, it is assumed that terminal transferase RNA half-lives are constant and independent of concentration. Support for this can be seen in studies involving mRNA degradation rates, as measured by the steady-state disappearance of Tdt specific mRNA upon removal of the inducing agents. Figure 3-14 is a representative Northern blot which depicts an "inducer removal" experiment, illustrating this Tdt mRNA disappearance. As graphically depicted in Figure 3-13, in the absence of inducers Tdt mRNA disappears from the induced cell with first-order kinetics (similar to the induction event). This figure also serves to reveal the reversible nature of the Tdt induction. Such a finding has never been previously reported. It appears that the reversibility observed probably derives from a transcriptional regulation of the gene. Using Figure 3-13, a steady-state approach to

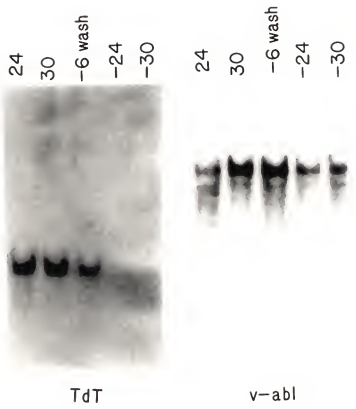


Figure 3-14. Reversibility of Tdt Induction

Cells were induced as described. At 24 hours post-induction, the culture was split in half, washed, resuspended in fresh medium plus or minus inducing agents, and cultured an additional 6 hours. Equivalent amounts of poly A^+ RNA were examined by northern blot analysis, using both (a) a Tdt-specific cDNA probe, and (b) an Abelson murine leukemia virus specific radio-labelled probe. Lanes designated 24 and 30 indicate continuous incubation in inducing agents for 24 or 30 hrs, while -24 and -30 represent uninduced cultures assayed at those times. Lanes marked -6 wash represent cultures where inducing agents were removed after 24 hrs, and cells cultured an additional 6 hours in medium containing no inducers.

evaluating Tdt mRNA half-life was undertaken. Based upon the observed mRNA decay curve, a $t_{1/2}$ for Tdt mRNA was calculated to be approximately 10-10.5 hr. This is the time required for one-half of the maximal steady-state induced level of Tdt mRNA to disappear following removal of the inducers. A more thorough discussion of this approach can be found in Chapter IV.

Based upon the data presented thus far, it appears that the increases in Tdt polypeptide synthesis and enzymatic activity result from marked increases in the accumulation of Tdt mRNA. In addition, the reversible nature of Tdt inducibility, previously demonstrated in protein and activity assays (Siden et al., 1985), was also seen in Tdt mRNA levels. The half-life of the Tdt mRNA in FLE cells following induction with dibutyryl cAMP and caffeine is 10.5 hr, as approximated by steady-state mRNA kinetics.

Analysis of In Vitro Nuclear Transcription During Induction

The technique of *in vitro* nuclear (or "run-off") transcription was used to assess the state of Tdt-specific gene expression in induced versus uninduced FLE cells. The system used was one utilized by Mather in the examination of immunoglobulin transcripts in lymphoid

cells (personal communication). Reaction conditions were optimized for such critical parameters as reaction time, temperature, salt concentrations (KCl and $MgCl_2$), and extraction technique. Conditions that yielded maximal total incorporation of ^{32}P-UTP were then assessed in terms of RNA transcript size using denaturing gel electrophoresis, and the reaction conditions described in Chapter II were utilized.

Radiolabelled transcripts from uninduced and induced FLE cells and from RL cells at various time points were isolated and hybridized to immobilized plasmids on either nitrocellulose or nylon membrane filters. The plasmids used in these initial studies included: pBR322 (as a negative control), pAB-u or p5 (plasmids containing immunoglobulin mu gene cDNA or genomic constant region sequences, respectively), pABsub3 (an Abelson murine leukemia virus probe), and pTdt (a Tdt cDNA sequence).

Positive results were obtained immediately with plasmids containing the Abl or the mu gene sequences, however the Tdt cDNA sequence routinely failed to elicit an autoradiographic signal. The pBR322 sequence was also negative for hybridization, as expected. Many parameters were altered in the hopes of finding conditions that favored Tdt gene transcription and allowed detectable hybridization. Experimental conditions were varied and

assessed methodically, using a matrix of differing salt, temperature and hybridization conditions. Cytosolic extracts from induced and constitutive Tdt-producing cell lines were also added to the nuclear reactions in an effort to detect Tdt nuclear transcripts, but proved unsuccessful. No alterations in the transcription reaction itself resulted in detectable Tdt-specific transcription, so the focus turned to hybridization conditions. Hybridization was performed at a variety of temperatures in both aqueous and formamide-based solutions, in the presence and absence of dextran sulfate, with plasmids bound to either nitrocellulose or nylon membranes, and under extremes of low stringency. Eventually, labelled transcripts did indeed hybridize with pTdt sequences, but only at a low level of stringency that also permitted non-specific pBR322 hybridization. A dot blot, which is representative of the numerous dot, slot, and Southern blotted experiments is shown in Figure 3-15. As seen in the autoradiograph, no pTdt hybridization is discernable (even after prolonged exposures). The pTdt plasmid is indeed bound to the filter, as shown in Figure 3-15 (b). Here, a filter that proved unsuccessful in a transcription assay was hybridized with the nick translated Tdt cDNA clone. The intense hybridization verifies that the plasmid was bound to the filter,

Figure 3-15. Analysis of In Vitro Transcription Products Using a Tdt cDNA Clone

(a) In vitro transcription reactions were performed using isolated nuclei from induced (15 hr) and uninduced FLE cells and ^{32}P-UTP as described. Labelled transcripts were isolated and hybridized to slot blots consisting of immobilized, denatured plasmids (Abelson leukemia virus DNA (abl), IgM mu heavy chain region DNA (mu), pBR322, Tdt cDNA (tdt)), and analyzed by autoradiography. (b) A filter utilized in (a) was stripped of all hybridized nucleic acid and rehybridized to ^{32}P nick-translated Tdt cDNA plasmid DNA to ensure that sequences were bound to filters. Cross hybridization is the result of common vector sequences in all four plasmids.

and accessible to a hybridization probe. The RNA isolation procedure was investigated as a possible source of mRNA degradation, yet the molecular weight range of transcripts obtained after both the in vitro transcription reaction and nucleic acid hybridization were found to be identical, and revealed no evidence of degradation as assessed by ethidium bromide-stained, methyl mercury gel electrophoresis (data not shown). The only interesting results obtained from this series of experiments concerned the expression of mu transcripts in the RL cell line, which is of T cell origin (Figure 3-16). The finding is not unique, however, as Kemp and coworkers (1980) have shown immunoglobulin transcripts to be present in many T cell and non-lymphoid cell lines.

A genomic Tdt clone was then obtained (kind gift of D. Baltimore) and used in the in vitro transcription assay. Representative results using this plasmid are shown in Figure 3-16. Once again, all positive and negative controls performed as expected, but this Tdt plasmid gave a positive result. In addition to hybridizing with transcripts from RL and induced FLE cells, the plasmid also reacted with uninduced FLE transcripts reproducibly, and to an equal degree. A preliminary conclusion from these data would tend to implicate a post-transcriptional regulatory mechanism for Tdt gene

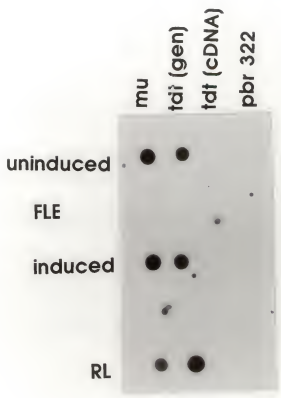


Figure 3-16. Analysis of In Vitro Transcription Products Using a Genomic Tdt Clone

In vitro transcription reactions were performed in the presence of ^{32}P-UTP using isolated nuclei from induced and uninduced FLE cells and RL cells as described. Labelled transcripts were isolated and hybridized to dot blots consisting of immobilized, denatured plasmids (IgM mu heavy chain region DNA (mu), genomic Tdt (gen), Tdt cDNA, and pBR 322), and analyzed by autoradiography.

expression. That is, the Tdt gene would be more or less constitutively transcribed in FLE cells, but as an extremely labile, short-lived mRNA. In cells that synthesize Tdt and possess enzymatic activity (RL and induced FLE cells), the mRNA would be stabilized, perhaps by cytosolic modification of a protein by cAMP. Such a hypothesis is not without precedent, although the number of reported genes regulated in this manner is small.

A key experiment was performed in order to eliminate the possibility of the genomic Tdt plasmid crossreacting with other gene products. The plasmid was nick-translated and used to probe EcoRI digested FLE DNA. The Tdt cDNA plasmid was also used as a probe. The results (not shown) revealed that the genomic clone hybridized to many sequences in the FLE cell genome, resulting in a "smear" in the lane, characteristic of repetitive DNA sequences, while the cDNA probe hybridized to five discrete EcoRI fragments. When the genomic Tdt probe was hybridized to Northern blots of fractionated nuclear and cytoplasmic RNA from induced and uninduced FLE cells (Figure 3-17 (b)), similar repetitive sequence smears appeared in both the nuclear RNA fractions. The cDNA probe, on the other hand, hybridized to only a single band in the cytoplasmic RNA fraction from induced FLE cells, corresponding to 2.1 kilobase mature Tdt mRNA. The fact that the cDNA probe

Figure 3-17. Detection of Tdt Transcripts in Nuclear and Cytoplasmic RNA Fractions from FLE Cells

Total nuclear and cytoplasmic RNA from induced (24 hr) and uninduced FLE cells were isolated, and 30 ug of each was electrophoresed through a 1% agarose gel, transferred to a nylon membrane filter, hybridized to various ^{32}P-labelled (nick-translated) probes, and analyzed by autoradiography. Labelled probes were removed completely after each exposure. Probes included a Tdt cDNA clone, a genomic Tdt clone, and a non-repetitive subclone derived from the Tdt genomic sequence.

cyto
nuc
ind
unind
nuc
cyto
TdT cDNA
cyto
nuc
ind
unind
nuc
cyto
TdT genomic
cyto
nuc
ind
unind
nuc
cyto
TdT intron

failed to hybridize to any nuclear precursor molecules, along with the absence of detectable hybridization in the in vitro nuclear run-off transcription experiments described above, may reflect the low steady-state levels of these precursors. It appears that the nuclear transcription experiment results were likely the result of repetitive sequences, and thus invalid.

A great deal of time and effort was spent in order to identify the repetitive sequences in the genomic Tdt plasmid, and to obtain a usable, non-repetitive genomic Tdt clone for use in the in vitro transcription assay. The task was complicated by the fact that no map of the clone was available. In order to expedite localization of the repetitive region(s), the clone was digested with the restriction enzymes EcoRI and BamHI in an overnight reaction. The digest resulted in the generation of seven fragments, one of which was the 2.6 kB vector, pUC-13. The pattern and approximate molecular weights of the fragments can be seen in Figure 3-18. The digestion products were electrophoresed through a 1% agarose gel, and transferred to a nylon membrane filter for hybridization. The probe used to detect repetitive sequences was nick-translated total FLE DNA. Genomic FLE DNA was isolated and radiolabelled as described in Chapter II. Specific activities were routinely over 1×10^8/ug.

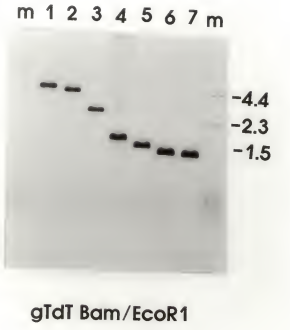


Figure 3-18. Restriction Pattern of Genomic Tdt Clone

A genomic Tdt clone (N. Landau, Whitehead Institute) was digested overnight with EcoRI and BamHI, and fragments were electrophoresed through a 1% agarose gel, extracted from the gel, labelled using the Klenow fragment of E. coli DNA polymerase I (BRL Co.) and α-^{32}P-ATP, and re-run through a 1% agarose gel as described. The gel was analyzed by autoradiography.

Hybridization conditions were identical to those used with RNA. By using a high specific activity probe, and exposing the autoradiograph for short periods of time, highly repetitive sequences could be readily differentiated from unique (low copy) regions. The hybridization pattern of these fragments can be seen in Figure 3-19 (c). The 1.6 kB fragment appeared to be highly repetitive, and was thus eliminated as a transcription probe. Other small fragments revealed varying amounts of repetitive sequence contamination upon prolonged exposure, so quantitative amounts of the largest bands of the EcoRI/BamH1 digest (approximately 4.3 and 3.9 kB, respectively) were isolated and digested with a panel of available restriction enzymes. This array of digests was blotted and hybridized with the nick translated FLE genomic DNA probe. Comparison of the autoradiograph obtained with a photo of the ethidium bromide stained gel prior to transfer (Figure 3-20), allowed rapid identification of fragments containing repetitive sequences. As seen in Figure 3-20 (b), the HaeIII digest of band 1 (4.3 kB) resulted in a lane that appeared to be free of repetitive sequences. In actuality, the HaeIII probably digested the repetitive region(s) of the fragment into many small fragments that either ran off the gel, or were left with regions of homology too small to allow hybridi-

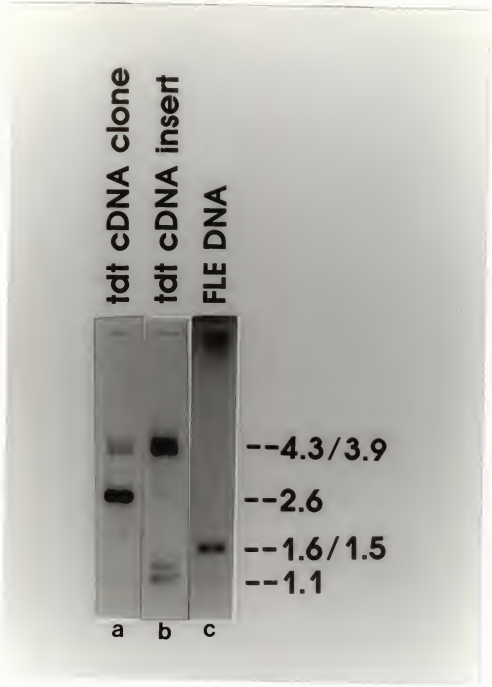


Figure 3-19. Assay for Repetitive Sequences in Genomic Tdt Clone

The genomic Tdt clone was completely digested with EcoRI and BamHI, and two ug of the digestion mixture were electrophoresed (per lane) through a 1% agarose gel, transferred to a nylon membrane filter, and hybridized to various nick-translated probes: (a) Tdt cDNA clone; (b) Tdt cDNA clone insert (same as (a) but with vector sequences removed); (c) FLE cell genomic DNA.

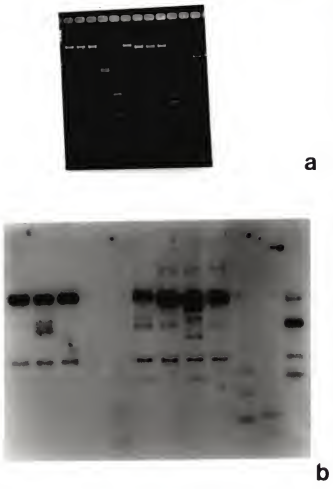


Figure 3-20. Identification of Non-Repetitive Sequences from Genomic Tdt Clone

The genomic Tdt clone was completely digested with EcoRI and BamHI. The largest fragment from the digest (4.3 kb, as seen in Figure 3-19) was isolated and digested with a battery of restriction enzymes. Two micrograms of each digest were electrophoresed through a 1% agarose gel, transferred to a nylon membrane filter, and probed with nick translated FLE genomic DNA in order to identify repetitive sequences. The autoradiograph (b) was compared to the ethidium stained gel (a) to identify non-repetitive fragments.

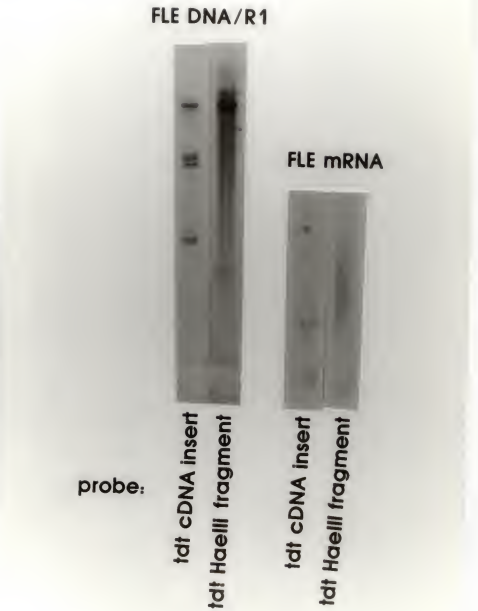
zation. The largest fragment of the HaeIII band 1 digest was a fragment of approximately 1.5 kB. Preparative quantities of this fragment were isolated, and used to probe EcoRI digested FLE cell DNA (Figure 3-21). One band was detected, which was one of the five to which the cDNA probe hybridized on the same blot.

The Hae III non-repetitive fragment was then analyzed by Northern blot analysis. Parallel lanes of induced FLE mRNA were hybridized to both the Tdt cDNA plasmid and to the Hae III fragment of the genomic clone. The former probe predictably hybridized with mature Tdt mRNA (approximately 2.1 kB), while the latter hybridized with a spectrum of RNA species compatible with intermediate sized transcripts representing various stages of mRNA processing. Interestingly, this pattern differs from the repetitive sequence "smear" seen in Figure 3-17 (b) in that the whole genomic probe smear extended the entire length of the lane, while the Hae III fragment probe apeared to have a rather distict beginning and end within the lane. The smallest mRNA molecules hybridizing to the Hae III fragment probe correspond to the approximate size of the mature mRNA species detected by the Tdt cDNA probe.

The Tdt cDNA probe did not hybridize to this HaeIII 1.5 kB non-repetitive genomic Tdt fragment (data not

Figure 3-21. Assessment of Non-Repetitive Sequence from Genomic Tdt Clone

(a) Genomic DNA from induced FLE cells was isolated, digested to completion with EcoRI, and ten ug per lane were electrophoresed through a 1% agarose gel and transferred to nylon membrane filters. The filters were probed with either nick-translated Tdt cDNA insert sequences or purified, non-repetitive genomic Tdt sequences, as indicated. (b) Poly A^+ RNA from induced FLE cells was isolated, and 10 ug per lane were electrophoresed, transferred to nylon membrane filters, and probed with the same probes as described above.


FLE DNA/R1
FLE mRNA
probe:
tdt cDNA insert
tdt HaeIII fragment
tdt cDNA insert
tdt HaeIII fragment

shown). It appears that the non-repetitive fragment of the genomic Tdt clone represents an intron. The fragment would still be usable as a probe for in vitro Tdt transcripts if it was an intron, since RNA splicing has not been demonstrated under the conditions used in the run-on nuclear transcription reactions (Marzluff and Huang, 1984).

The fragment was then subcloned in the pUC-8 vector, and tested in the nuclear transcription assay. Once again, all controls functioned as expected, but no signal was obtained using the Tdt intron probe (Figure 3-22). Exhaustive alterations of hybridization conditions again failed to elicit a Tdt-specific signal, and at this point it was decided to terminate this aspect of the project.

Testing of Other Inducing Agents

Biologically relevant compounds that might act as triggers for the induction of Tdt gene expression were carefully selected, and tested under conditions that had previously elicited physiological responses. The Tdt-inducible FLE cell line provided an assay for signals that may act as primary triggers for the enzyme's expression. Agents were selected based upon suitability as in vivo mediators of pre-B cell differentiation. The

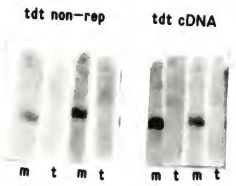


Figure 3-22. Analysis of In Vitro Transcription Products Using a Non-Repetitve Tdt Intron Probe

In vitro transcription reactions were performed in the presence of ^{32}P-UTP using isolated nuclei from dibutyryl cAMP/caffeine induced (16 hr) and uninduced FLE cells and RL cells as described. Labelled transcripts were isolated and hybridized to blots of insert fragments from plasmids containing either mu immunoglobulin constant region (m), non-repetitive genomic Tdt sequences, or Tdt cDNA sequences (both denoted by "t") as indicated, and analyzed by autoradiography. Duplicate hybridizations are presented for each of the Tdt clones used.

agents tested were classified as either DNA synthesis inhibitors, cellular stress modulators, or hormonal mediators. The concentrations tested represented a range that had been shown to be appropriate for lymphoid cell responses. In addition, most of the compounds were assayed for the inducibility of Tdt gene expression at several different times after addition. The results of these experiments are summarized in Table 3-1. A further discussion of the implications and rationale for these experiments is presented in Chapter IV.

Inhibitors of DNA synthesis were tested based upon the well-documented exclusivity of cellular differentiation versus proliferation (Lotem and Sachs, 1982). The somatic changes supposedly mediated by the action of Tdt occur at a time of cellular differentiation associated with DNA strand rearrangement (Alt and Baltimore, 1982). It was hypothesized that the inhibition of cellular proliferation may force the FLE cell into a differentiative mode, and possibly evoke the expression of Tdt. The DNA synthesis inhibitors that were utilized included hydroxyurea, parabactin, and bromodeoxyuridine. They all inhibit DNA synthesis indirectly, via an inhibition of ribonucleotide reductase (Kornberg, 1980; Cavanaugh et al., 1985; Meuth and Green, 1974).

TABLE 3-1: Inducing Agents Screened

Inducers [optimal]	Non-Inducers [highest concentration tested]
dbcAMP [1 mM]	xanthine [0.33 mM]
caffeine [2.6 mM]	theobromine [0.28 mM]
theophylline [2.8 mM]	3-methyl xanthine [0.03 mM]
8-bromo cAMP [1 mM]	dbcGMP [1 mM]
ATP [1 mM]	sodium butyrate [2 mM]
BUdR [20 ug/ml]	GTP [10 mM]
Hydroxyurea [1 mM]	CTP [10 mM]
Parabactin [5 uM]	TTP [10 mM]
	TPA [1 pg/ml]
	retinoic acid
	heat shock ($42^{o}C$, 10')
	gamma-irradiation (2000 R)
	PGE_1 [10^{-5} M]
	IL-2
	IL-3

The inhibitors of DNA replication proved to be effective inducers of Tdt gene activation. Representative Northern blot analyses of Tdt-specific mRNA are illustrated in Figures 3-23 and 3-24. The efficacy of the agents to inhibit DNA synthesis was assessed using viable cell counts and ^{3}H-thymidine incorporation throughout the course of the experiments. These data are presented in Figures 3-25 and 3-26, respectively. The agents proved to be potent inhibitors of DNA replication in all cases. Cell growth was assessed over the first third of the induction period, and revealed marked toxic effects of a few tested agents. The hydroxyurea proved quite toxic to the FLE cells both at concentrations above 1 mM, and for periods of incubation exceeding 12 hr (data not shown). The lane in Figure 3-24 contains RNA from the viable FLE cells (approximately 12%) cultured in 1 mM hydroxyurea for 16 hr and is not appropriate for comparison to other lanes.

A fluorescence activated cell sorter analysis (FACS; flow cytometry) was carried out on FLE cell cultures treated with various Tdt inducers, as a more precise monitor of S phase activity. These data are illustrated in figs 3-27. A comparison of dibutyryl cAMP/caffeine induced FLE cells with untreated cells revealed no marked differences in cell cycle kinetics, precluding a role for

Figure 3-23. Tdt mRNA Levels in FLE Cells Following Treatment with Prostaglandin E_1 and BUdR

FLE1-4 cells were cultured for 24 hrs in the presence of various concentrations of PGE_1 or BUdR as noted. Poly $(A)^+$ RNAs were isolated, denatured with glyoxal, electrophoresed through a 1% agarose gel, transferred to a nylon membrane filter, and hybridized to a radiolabelled Tdt specific cDNA probe. Ten micrograms of each sample were analyzed per lane, except RL, which represents 5 ug of RL cell poly $(A)^+$ RNA. Lane assignments are: a, 10^{-7} M PGE_1; b, 10^{-6} M PGE_1; c, 0.2 ug/ml BUdR; d, 2.0 ug/ml BUdR; e, 20 ug/ml BUdR; f, uninduced cntl; g, dbcAMP/caffeine (0.1 mM each).

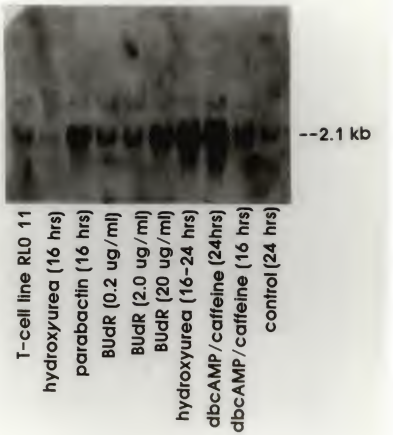


Figure 3-24. Tdt mRNA Levels in FLE Cells Following Treatment with DNA Synthesis Inhibitors

FLE1-4 cells were cultured in the presence of various DNA synthesis inhibitors for various periods of time up to 24 hr (as indicated). Bromodeoxyuridine-treated cells were cultured for 24 hr. Poly $(A)^+$ RNAs were isolated, denatured with glyoxal, electrophoresed through a 1% agarose gel, transferred to a nylon membrane filter, and hybridized to a radiolabelled Tdt specific cDNA probe. Ten micrograms of each sample were analyzed per lane.

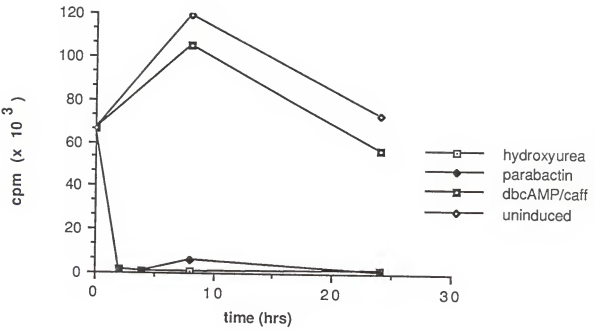


Figure 3-25. FLE Cell ^{3}H-Thymidine Incorporation in the Presence of Various DNA Synthesis Inhibitors

FLE cells were cultured in the presence of various DNA synthesis inhibitors as indicated (concentrations as in Table 3-1). Triplicate aliquots were removed at various time points, labelled with 5 uCi of ^{3}H-Thymidine for 60 min, washed and filtered. Dried filters were assayed by liquid scintillation counting, and the results plotted as shown. Standard deviations for each point plotted never exceeded 18%.

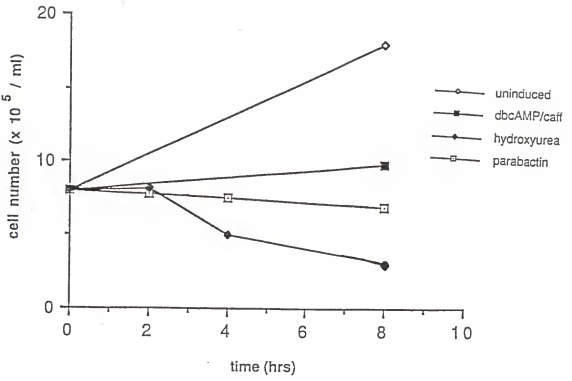


Figure 3-26. Kinetics of Cell Growth During Tdt Induction in FLE Cells

FLE cells were cultured in the presence of various DNA synthesis inhibitors as indicated (concentrations as in Table 3-1). Aliquots were removed at various times, and viable cell counts were determined using trypan blue dye exclusion. Three samples were counted per time point, and values obtained never varied by more than 8%.

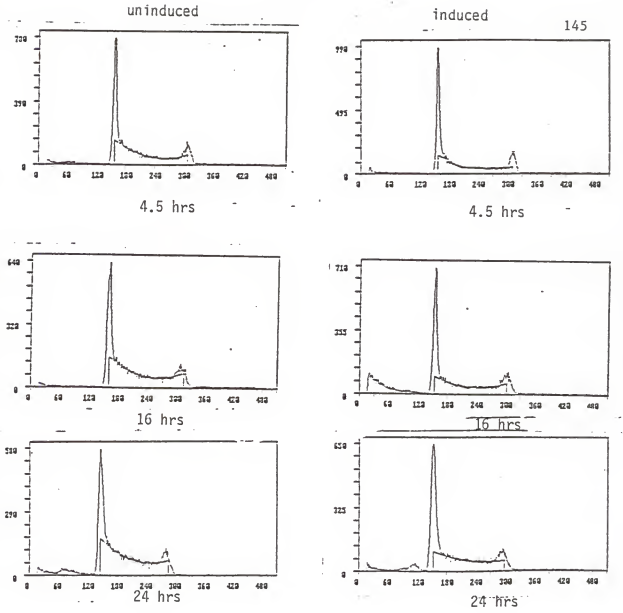
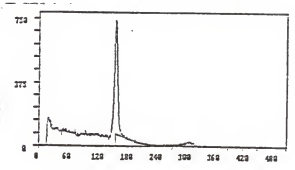
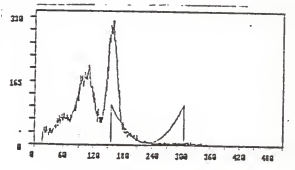


Figure 3-27. Fluorescence Activated Cell Sorter Analysis of Induced FLE Cells

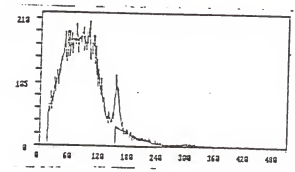
Aliquots of FLE cells which had been cultured in the presence of various inducing agents for indicated periods of time were adjusted to 50% ethanol, stained with propidium iodide, and analyzed with a FACS II flow cytometer (Becton-Dickinson) interfaced with a microcomputer (Hewlett Packard 45B). DNA distributions were obtained from analyses of the red fluorescence from the PI-stained DNA, and were determined through the kind assistance of Michael Ingeno (University of Florida).



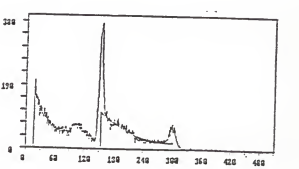
hydroxyurea 4.5 hrs



hydroxyurea in at 16 hrs, assayed at 24 hrs



hydroxyurea 16 hrs



parabactin 3.5 ul 16 hrs

Figure 3-27--continued

cell cycle regulation of Tdt expression. Although the DNA synthesis inhibitors profoundly inhibited DNA replication, the cell cycle data similarly revealed a lack of correlation of Tdt transcription with phase of the cell cycle. This figure also demonstrates the cytotoxic effects of hydroxyurea on FLE cells after 16 hours of culture.

Adenosine 5'-triphosphate (ATP) was also tested as a possible Tdt-inducing agent. The rationale for testing this compound related to the fact that ATP is a potent inhibitor of Tdt in vitro. It has previously been shown that in the case of the dihydrofolate reductase (DHFR) gene, for example, the addition of a competitive inhibitor (such as methotrexate) will enhance DHFR gene expression via a mechanism known as gene amplification (Alt et al., 1978). It was hypothesized that the expression of Tdt may likewise be susceptible to such a mechanism by the addition of an inhibitor. Adenosine 5'-triphosphate was indeed found to induce the synthesis of Tdt, as seen in Figure 3-28. This reproducible effect was seen at ATP concentrations of 0.25, 0.5, and 1.0 mM, after a 24 hr incubation period.

Agents such as heat shock, ethidium bromide, and gamma irradiation are all considered cellular stress agents, and evoke activation of cellular repair mechan-

Figure 3-28. ATP Induced TdT Expression

FLE cells were grown for 24 hours in medium supplemented with either 0.5 or 1.0 mM ATP. Aliquots were biosynthetically labelled with 50 uCi/ml ^{35}S-methionine, immunoprecipitated, and analyzed by SDS PAGE as described. No background Tdt was seen in uninduced control FLE cells (data not shown). Marker lane contains Tdt from immunoprecipitated RL cells.

isms (Kornberg, 1980). They were tested as possible inducers of Tdt expression based upon a presumption that the enzyme may represent a component of a repair system utilized by the cell in cases of extreme DNA damage. Neither heat shock (42^{o} C for up to ten minutes), ethidium bromide (200 uM), nor gamma-irradiation (up to 2000 rads) were shown to induce the accumulation of terminal transferase mRNA in FLE cells (data not shown).

Hormonal and growth factor influences upon cell differentiation and maturation are complex and often rely upon the interactions of cells from disparate lineages. In mammals, prostaglandins of the E series (particularly PGE_1) have been found in the highest concentrations in the thymus gland, the richest source of Tdt (Goldschneider, 1982). In addition, prostaglandins had previously been shown to increase levels of intracellular cAMP in lymphocytes (Scheid et al., 1978), a condition shown to be associated with Tdt expression (Siden et al., 1985).

Negative data were obtained when several hormonal mediators of lymphoid cell differentiation and proliferation were tested. Interleukins 2 and 3, cis-retinoic acid, prostaglandin E-1 (PGE_1), and a phorbol ester (12-O-Tetradecanoylphorbol 13-acetate, or TPA) all were incapable of Tdt induction. Figure 3-23 illustrates a

Northern blot containing two poly A^+ RNA samples from cells exposed to PGE_1, a known activator of cAMP, which appeared to show no induction of Tdt mRNA. This compound was also tested at three other concentrations (not shown) and proved to be negative for the induction of Tdt over a six log range of concentrations (10^{-6} to 10^{-12} M).

Intracellular cAMP Concentrations After Induction

Previous work had revealed that agents which were known to increase intracellular cAMP levels also induced the expression of Tdt in certain pre-B cell lines (Siden et al., 1985). A preliminary analysis of cAMP concentrations in FLE cells cultured in the presence of hydroxyurea was undertaken in order to investigate whether those inhibitors of DNA synthesis which induced Tdt expression acted through a similar second messenger system.

Intracellular cAMP concentrations were assayed following Tdt induction of FLE cells with hydroxyurea using a commercially available kit (see Chapter II), following a non-acetylated sample protocol. The standard curve (Figure 3-29) was linear between 25 and 1600 fmol cAMP, which was the effective range utilized for the unknown samples. Intracellular cAMP levels were deter-

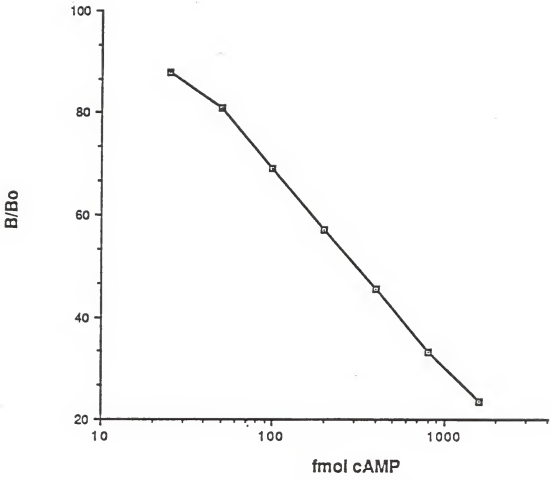


Figure 3-29. Cyclic AMP Standard Curve

Commercially prepared standards were assayed in a cAMP Radioimmunoassay System (Amersham Co.), as described in text. Samples were assayed in triplicate, and the mean value was used to calculate the ratio of bound/free ligand obtained was plotted. Standard deviations for any given point did not exceed 15%.

mined at various times following exposure of FLE cells to hydroxyurea, and the results graphically represented in Figure 3-30. Two dilutions were assayed for each sample, and three replicates were performed per dilution. The mean results of the radioimmunoassay were then interpolated onto the standard curve, and finally converted to pmol cAMP per 10^7 cells.

As shown, a significant increase in intracellular cAMP was seen in the presence of hydroxyurea. This "spike" is seen at approximately 2 hr after addition of hydroxyurea, and precedes the appearance of detectable Tdt mRNA by approximately 6 hr (data not shown). Cell viability was determined throughout this eight hour time course by trypan blue exclusion, and was never below 73%. Intracellular cAMP levels in dibutyryl cAMP-induced FLE cells were ambiguous, owing to an immunological cross reactivity between endogenous and derivitized (dibutyryl) cAMP with respect to the supplied antiserum (not shown).

These data, in association with data describing other agents effecting Tdt expression in FLE cells (above) lead to the conclusions that several agents that inhibit cellular proliferation (or more specifically, DNA synthesis) also appear to induce Tdt. Moreover, there may be a transient cAMP induction associated with growth inhibition in these cells.

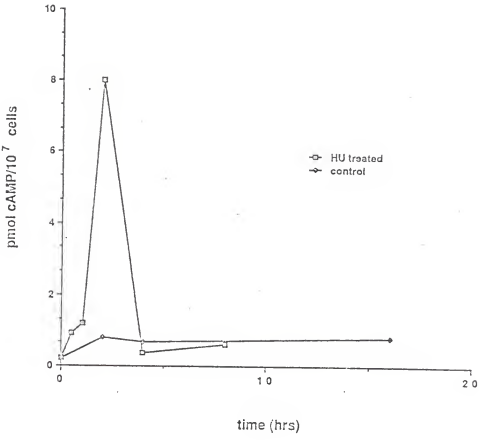


Figure 3-30. Kinetics of Intracellular cAMP Accumulation in Hydroxyurea Treated FLE Cells

FLE cells were cultured in the presence or absence of 1 mM hydroxyurea. At various times indicated, aliquots were withdrawn, quick-chilled, centrifuged, and cell pellets were assayed for intracellular cAMP content as described in Chapter II. Control values represent uninduced FLE cells. Cells used in this experiment were all over 73% viable, as determined by trypan blue dye exclusion.

Phosphorylation Patterns in Induced Cells

The role of protein phosphorylation in cyclic AMP-mediated eucaryotic gene expression is well established (reviewed in Chapter I). Particularly noteworthy is the reported phosphorylation of histone H1 following intracellular increases in cAMP concentrations. This phosphorylation has been reported to result in the induction of new RNA species (Hohmann, 1983). In order to determine whether Tdt induction of FLE cells may operate through a similar mechanism, induced and uninduced FLE cells (at 15 hrs post-induction) were metabolically labelled with ^{32}P for 2-2.5 hrs as described (Chapter II), and histones were isolated and analyzed by SDS-PAGE. Representative data are shown in Figure 3-31. The position of histone H1 was ascertained by comparison with acid-extracted unlabelled histone standards (kind gift of L. Green).

A prominent radiolabelled species can be seen in the lane from the induced cells in the figure. The band corresponded with the migration of unlabelled histone H1 in the marker lane, and disappeared in samples pretreated with proteinase K prior to electrophoresis (not shown). As expected, neither DNAse nor RNAse were active against the labelled histone. Samples were passed over a DE-52

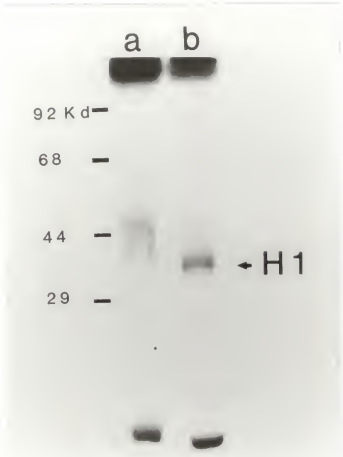


Figure 3-31. Phosphorylation of Histone H1 in Induced FLE Cells

Induced (dibutyryl cAMP and caffeine, 0.1 mM each) and uninduced FLE cells (1.5×10^6) were labelled at 20 hrs post-induction with $H_3{}^{32}PO_4$ for a two hour period, and histones were acid extracted as described (Chapter II). Phosphorylation was determined by PAGE of extracted proteins, and histone H1 was identified using unlabelled standards. Lane (a) was from uninduced cells, and lane (b) was obtained from induced cells.

column to remove any residual nucleic acid, with no loss of band intensity. The concentration of acid-extracted protein in both induced and uninduced cell preparations was very similar, as determined by Bradford analysis (data not shown).

CHAPTER IV

DISCUSSION

Introduction

The goals of this study were to investigate the induction and regulation of the expression of the murine terminal deoxynucleotidyl transferase gene. Based upon data presented herein, it appears that the modulation of the Tdt gene is associated with a change in Tdt mRNA content in the inducible cell. The Tdt-specific mRNA levels and immunoprecipitable Tdt protein both correlate well with functional Tdt activity in induced cells. Induction of Tdt in "susceptible" lymphocytes had previously been linked to cAMP by several pieces of evidence (Siden, et al. 1985), the foremost of which related to the nature of the inducing agents. Dibutyryl cAMP, an agent which mimics naturally produced cellular cAMP (yet is able to transverse the cell membrane with no degradation or loss of activity), had previously been shown to be an effective agent to activate Tdt biosynthesis. A synergistic effect was noted with the addition of caffeine (or various other substituted xanthines). This effect has been postulated to be a

result of the potent phosphodiesterase-inhibiting activity of the compounds, allowing for the intracellular accumulation of cAMP. The present study has demonstrated a modulation of Tdt gene expression following the addition of agents which interfere with efficient DNA replication.

The importance of investigating Tdt gene expression and regulation transcends basic science, into the realm of applied studies. The fact that Tdt is a marker gene for acute lymphocytic leukemia (ALL) adds import to any conclusions. A disease marker, by definition, should be diagnostic for a given condition. Inherent in such a definition is the assumption that the marker be found on none, or at least very few normal cells bearing similar phenotypes, or located in the same microenvironmental compartment. Hence the presence of a myeloma would be difficult to ascertain based solely upon the presence of immunoglobulin-containing cells in the peripheral blood. The finding of Tdt^+ pre-B cells in the peripheral blood of an individual, on the other hand, is extremely uncommon. In fact, Tdt has never been detected in the periphery of a normal individual; even in the bone marrow, only 3% of all cells have the Tdt^+ phenotype (Janossy et al., 1982). While the absence of Tdt^+ cells

does not exclude the diagnosis of ALL, its presence is more than suggestive.

The fact that the normal counterpart to the transformed cell in Tdt^+ leukemia is present in such low numbers among blasts in the marrow leads to interesting and compelling speculation regarding the leukemogenic process and Tdt expression. Clearly, as ALL is the most common malignant state seen in children, and Tdt positivity is seen in a majority of these cases (95%), a causal link can be postulated. Non-transformed Tdt^+ blasts are normally in the minority, yet here is a disease state in which these few cells are "selectively" transformed. This observation precludes a stochastic model for leukemogenesis. Roughly ninety-seven per cent of childhood leukemias should be Tdt^- if the leukemogenic event was random. The spectrum of phenotypes of transformed cells in clinical isolates of ALL would theoretically resemble the spectrum of normal cell phenotypes in the bone marrow. Instead, there seems to be some predilection for immature Tdt^+ lymphocyte transformation. Either the cells which are initially transformed in ALL are Tdt^-, in which case the leukemogenic event triggers Tdt expression, or the few Tdt^+ cells are exquisitely sensitive to the transforming event. If the first case is true, the Tdt induction model affords an *in vitro* analysis of the

triggers which the leukemogenic event may provide *in vivo*. Stated more precisely, the initial leukemogenic event, or some gene product being continuously produced as a result of it, supplants the cAMP-dependent step required *in vitro*. If the latter hypothesis is correct, then the inducing system provides the means to investigate exactly what changes the presence of Tdt creates; what events provide the ideal milieu for cellular transformation. These provocative extrapolations are all approachable with the inducible system which we are utilizing, however the first processes which must be understood are those of Tdt gene expression and regulation. To that end, this project has been undertaken.

Determining the level (or mode) of regulation of a particular gene is a necessary prerequisite to the study of its control. Although the vast majority of eukaryotic genes studied thus far have exhibited transcriptional regulation, the number of systems using posttranscriptional and translational control is not insignificant. Conditional mutants have been invaluable in studies of gene regulation, especially in procaryotes. The inducible system for terminal transferase (Siden et al, 1985) is analogous to a conditional mutant, in that the conversion of a "wild type" to an "induced" cell is a reversible phenotypic change achieved within a cloned cell popu-

lation by altering certain growth conditions. Furthermore, this system represents the only inducible system for the enzyme in a cloned cell population. This avoids complications arising from Tdt induction of heterogeneous populations of cells from many different lineages and stages of differentiation (Pazmino et al, 1978; Cayre et al, 1981). Thus, induction of the enzyme in established cell lines provides the most valid condition with which to determine the mode of Tdt gene regulation and potential *in vivo* function.

Tdt mRNA Studies in Induced FLE Cells

The attempt to clone the terminal transferase gene was an essential first step in assaying gene expression. The strategy employed for this cloning, subtractive hybridization, had previously been proven effective in the cloning of the dihydrofolate reductase gene (Alt et al., 1978), as well as immunoglobulin J chain gene (Mather et al., 1981). Although this aspect of the project was not completed, it did lead to the translation and immunoprecipitation of full length Tdt protein from a population of heterogeneous mRNAs.

Fractionated mRNA from the RL constitutive Tdt-producing line was added to a rabbit reticulocyte *in*

vitro translation system and optimized for maximum incorporation. The products of the translation were then immunoprecipitated, and successful precipitation of full-sized murine Tdt was achieved. This result had not been reported previously in the literature. Numerous attempts to demonstrate functional activity from this *in vitro* Tdt, including phosphocellulose purification of the translation mix, proved fruitless. The sensitivity of the activity assay was hampered by an endogenous, reproducible, primer-dependent polymerase activity present in the reticulocyte preparation. Translation of Tdt-containing mRNA in frog oocytes also proved refractory to activity assays. An obvious limitation to either translation system is the minute amount of Tdt protein produced.

The analysis of steady-state Tdt mRNA levels after induction provided the first clue as to the level of regulation of the Tdt gene. The accumulation of Tdt mRNA, while not precluding secondary levels of control, strongly implicated transcriptional regulation. Transcriptional control has been demonstrated in a substantial number of gene systems analyzed, possibly surpassing both post-transcriptional and translational regulatory mechanisms. These terms are purely descriptive, as it is difficult to attribute the regulation of a gene solely to

one metabolic process. Rather, all processes represent a continuum, separable only by the arbitrary constraints employed to simplify the ways in which we interpret them.

Prior to the advent of in vitro transcription systems, many workers relied upon a steady-state approach to the transcriptional analysis of gene expression. Most of the mathematical approaches to mRNA concentration and half life analyses have been compiled by Rodgers and co-workers (1985). The change in the concentration of a hormonally-controlled mRNA transcript can be described by

$$dC/dt = k_s - L\,C \qquad (1)$$

where k_s is the rate of mRNA synthesis, and L is a first-order rate constant of mRNA degradation. The maintenance of uniform induction conditions for sufficient periods of time enables one to achieve a steady-state system where $dC/dt = 0$. Thus, equation (1) simplifies to

$$C_{steady\text{-}state} = k_s/L \qquad (2)$$

This analysis formally states the obvious, and demands acceptance of a kinetic model, but put simply, when Tdt expression has reached a steady-state, which in this case has been shown to be at approximately 30 hrs post-

induction (figure 3-13), the rate of Tdt-mRNA synthesis equals its degradation rate. In fact, the same graph can be utilized to arrive at a value for the degradation of the mRNA, relative to the rate of synthesis. The mathematics involved assumes a first-order rate of mRNA degradation, which appears consistant with the observed linearity of the data seen after withdrawl of the cAMP/caffeine inducer. By assigning arbitrary units to the graph depicting the kinetics of Tdt mRNA accumulation (based upon densitometric tracings of Northern blots), relative biosynthesis and degradation slopes (k_s and L, respectively) can be determined, and more importantly, an approximate value for the Tdt mRNA half-life can be calculated. After removal of the inducing agents, it required approximately 10 hrs for one half of the steady state Tdt mRNA to disappear. Further analyses of the degradation rate of a hormonally-stimulated mRNA are difficult to perform, since the induction event could be acting to inhibit the synthesis of a molecule responsible for mRNA degradation, or even a molecule which simply inactivates the degrader. The complexities which can develop are immense, so as to preclude overinterpretation of the data.

Thus it appears that the Tdt RNA molecule is both induced and degraded by first-order kinetics. The

accumulated message reaches equilibrium shortly after 30 hours of induction, and this level can be maintained indefinitely in the presence of appropriate inducers. Upon removal of the inducing agents, the Tdt mRNA decays rapidly, returning to a basal (uninduced) level of expression in approximately 12 hours. The induction event is reversible, since the cells can be successfully restimulated following such an inducer removal, and similar maximum levels of expression can be reachieved (as described in Chapter III).

Nuclear Transcription of Tdt

The *in vitro* transcription experiments designed to assess the level of molecular regulation of the Tdt gene proved inconclusive. Initial experiments using the Tdt cDNA clone revealed non-specific hybridization at the same low level of stringency which resulted in hybridization to pBR322 sequences. No modifications to either the transcription reaction or hybridization conditions altered this negative result. The explanation for such a finding is difficult to imagine. The explanation for such results was considered in terms of two parameters.

Hybridization and reaction conditions are always suspect in this type of experiment. The fact that the

Tdt gene is large is apparent from an analysis of the size range of FLE cell EcoR1 fragments which hybridize to the Tdt cDNA probe (figure 3-21). A rough, minimum estimate of the gene size, based upon this blot plus data documented by Landau et al. (1985) is approximately 25 kB. It is possible that, if the number of introns in such a gene is extremely large, the cDNA may not contain ample stretches of homology to effect binding to the transcript. It has been found that the first intron in the Tdt gene is only 50 nucleotides in length (N. Landau, personal communication). Also, while the in vitro transcription reaction was painstakingly optimized for parameters such as salt and substrate concentrations, using both total incorporation of UTP and the spectrum of mRNA lengths obtained as assays, it is always possible that the reaction had a pause or stop sequence which made it refractory to nucleotide incorporation. This could be due to a structural anomaly in the Tdt gene itself, resulting in attenuated transcripts. Perhaps only a portion of the gene was labelled. The isolation procedure itself was analyzed in terms of possible mRNA degradation, and the molecular weight range of transcripts obtained was unchanged after the hybridization procedure (data not shown). The fact that both Abelson virus-specific and immunoglobulin mu constant region

probes, representing mature messages of approximately the same size, performed well in the system also argue against such explanations. The description of the Tdt gene structure is no doubt forthcoming, and may be helpful in addressing these concerns.

A second possible explanation for the failure to detect Tdt-specific gene transcripts using either the cDNA or the intron probe concerns the requirements for in vitro nuclear transcription of several genes. Previous reports have documented the requirement of either cytosolic factors (Gorski et al., 1986) or heavy metal ions, such as zinc (N. Heintz, personal communication), in successful transcription of certain genes. During the course of these experiments, several transcription reactions were performed in the presence of cytosolic extracts from induced and even constitutive Tdt-producing cell lines. Many concentrations were tested, but the gene still proved elusive to transcription in vitro. The heavy metal requirement was not tested, however, and still remains a possibility.

Identification of Tdt Inducing Agents

The analysis of eukaryotic gene expression reveals a complexity, owing largely to a lack of consistent

regulatory paradigms between gene families. Structural elements such as strong consensus promoter regions and enhancer sequences have been identified in several genes. The fact that most of these regulatory regions are tissue or lineage specific adds another level of complexity to a unified model of gene expression. The stimuli to which these regulatory elements respond are even more difficult to define. The search for biologically relevant modulators of gene expression is complicated by the seemingly infinite variety of microenvironments to which a cell is exposed. Even within a given tissue microenvironment, developmental stage-specific gene expression may be controlled by differential sensitivities to locally produced growth factors, as is the case in the mammalian liver (Clayton et al., 1985a, 1985b).

The search for biologically relevant agents which trigger Tdt synthesis was arduous. While it can be argued that cAMP acts as the second messenger in Tdt production, a mechanism shared by many hormonally regulated genes, the assigning of a substance in a role as a primary messenger is more difficult to verify. The agents which were tested in this regard (Table 3-1) were chosen carefully, with distinct hypotheses in mind.

Trials of DNA replication inhibitors were based upon the premise that cells will exclusively choose one of two pathways at any given point in time; either differentiation or proliferation. Inherent in such a view is the fact that these stages are likely mutually exclusive. Quiescience may in fact represent a period of cell transition between such pathways. Several elegant studies (Lotem and Sachs, 1982; Symonds and Sachs, 1983) have demonstrated distinct factors governing each of these states in the myeloid lineage. If Tdt is indeed involved in the generation of antigen diversity at an early, antigen-independent stage (Alt and Baltimore, 1982), then the cell is probably in a non-proliferative, or differentiative stage of development. This is in concordance with a simplistic yet supportable theory of cAMP regulation of cell growth. Namely, the fact that rapidly proliferating (normal or transformed) cells have depressed cAMP levels (and often increased cGMP levels). The converse has been seen in differentiating, non-dividing cells: an increase in cAMP, and a depressed cGMP content (Hait and Weiss, 1976).

Agents such as parabactin, 5-bromodeoxyuridine, and hydroxyurea, which inhibited DNA synthesis in this study (presumably due to an inhibition of ribonucleotide reductase) all elicited Tdt induction in FLE cells. The

importance of such a finding will be considered in light of the nature of these agents and their mechanism of action.

Parabactin is a spermidine catecholamide iron chelator synthesized and elaborated by the plant pathogen P. dentrificans (Cavanaugh, Jr. et al., 1985). The compound has been shown to inhibit the growth of L1210 cells in vitro, as well as inhibiting the replication of several DNA (but not RNA) viruses. There is not a large body of information available about parabactin, owing to the recency of its isolation, but these researchers have determined that the effects of parabactin seem to result from an inhibition of ribonucleotide reductase.

Bromomodeoxyuridine (BrdU) is a nucleotide analog of thymidine, which has been used extensively to induce cellular differentiation, viral multiplication and various mutations (Meuth and Green, 1974). While many effects of BrdU may be due to the incorporation of the compound into replicating DNA strands, these workers have demonstrated that BrdU is a potent inhibitor of ribonucleotide reductase.

Hydroxyurea has been shown to interrupt E. coli and phage T4 ribonucleotide reductase in vitro by inactivating the organic free radical of the B2 subunit

of the enzyme. It is also active in animal cells (Thelander and Reichard, 1979). Resistance to hydroxyurea in animal cells is manifested by the overproduction of ribonucleotide reductase. Basically, hydroxyurea acts to block production of all deoxyribonucleotides, and hence DNA synthesis. The compound, which is known to kill cells in the S phase of the cell cycle with prolonged exposure or high dose, was previously shown to cause a 40-70% increase in the number of B cells which can be cultured from adult bone marrow cells in a 24 hr in vitro assay (Le Bouteiller et al., 1982). This phenomenon was noted only in cell populations from organs containing pre-B cells (as opposed to lymph nodes or spleen). It has been suggested that in the immune system, hydroxyurea kills cells mediating supression of differentiation (Le Bouteiller et al., 1982)

The fact that all these agents act to induce the synthesis of Tdt implies that Tdt may be synthesized in pre-B lymphocytes in the absence of DNA replication. One may then speculate that some early stages of B-cell differentiation, and thus antibody diversity may be linked to clonal inhibition, and envision a multi-step nature to the acquision of immunologic diversity. The first stage, the generation of N-region diversity occurs during the immunoglobulin gene rearrangement stage of

pre-B cell differentiation. This is the point at which Tdt probably exerts its influence. Perhaps the enzyme is linked, or coexpressed with a lymphoid-specific recombinase, such as the one proposed by Yancopoulos et al. (1986). It would be interesting to determine whether such a protein could be synthesized in the absence of DNA synthesis, as well. Perhaps this early stage of immunoglobulin gene rearrangement takes place in quiescent cells. The generation of an in vivo signal which inhibits cell division (and DNA replication) in pre-B lymphoid cells in the bone marrow or fetal liver, might allow differentiation proteins and enzymes to be synthesized. A convenient way to effect such a change exclusively in those cells mature enough (or of the correct lineage) without affecting other cells would be to utilize a specific cell surface receptor, such as one for an interleukin, prostaglandin, or other hormone. The receptor could be used to specifically stimulate intracellular cAMP in suitable cells, and begin the phosphorylation cascades which may be the key to activating the immunoglobulin gene locus rearrangement mechanisms.

The second stage of immunoglobulin diversity acquisition is found later in the B cell differentiative pathway, and is probably linked to clonal proliferation. This phase is noted by the appearance of somatic point

mutations, a modifier of antigen-antibody affinity. These point mutations have been well documented in both B and T cells (Weigert et al., 1970; Kim et al., 1981; Gearhardt et al., 1983; Barth et al., 1985). Once the B cell has successfully rearranged its immunoglobulin locus, and carried out its "non proliferative" (DNA synthesis "unlinked") diversification, it is quiescent until antigen is encountered. At this point, clonal proliferation and the class switching event take place, with their associated point mutations.

There is an interesting corroborative study concerning the regulation of Tdt in murine thymocytes, which was carried out by Rothenberg and Triglia (1983). They examined the relationship between Tdt synthesis and proliferation of thymocytes in two different ways: by comparing Tdt production in adult thymocytes, which are largely quiescent, to neonatal thymocytes, which represent a more rapidly dividing population; and by comparing the level of Tdt synthesis in proliferating verus growth-arrested cell populations in the murine thymus. Neonatal and late-fetal thymocytes were shown to synthesize Tdt at much lower levels than thymocytes from weanlings or adults, as measured by immunoprecipitation of biosynthetically labelled proteins. Perinatal thymocytes, known to be a highly proliferating cell popula-

tion, did not demonstrate correspondingly high Tdt synthesis. Adult proliferating cells do make some Tdt, however the majority was synthesized by cortical thymocytes which showed terminally arrested growth (these are the small, post-mitotic cortical thymocytes, as opposed to larger, proliferating cortical lymphoblasts). They concluded from this that Tdt synthesis was uncoupled from cell proliferation.

The group also performed in vitro studies on murine thymocytes, and showed that the neonatal cells increase their Tdt synthetic rate when removed from the thymic microenvironment and placed in culture. Their experiments determined that conditions which stimulate Tdt synthesis in vitro are not associated with mitogenesis, but rather with growth arrest. Further, agents which were shown to enhance T cell proliferation (such as IL-2) had no effect upon Tdt production (Tdt production was in fact supressed in many cases). Combined with data from other workers which showed that Tdt expression is unaffected by cell cycle stage in continuously growing leukemic cells (Harrison et al., 1976), it appears that in the thymus, where the major concentration of Tdt is seen, high levels of de novo Tdt synthesis continues, even in the irreversible growth arrest pahse of most adult cortical thymocytes. It thus appears that Tdt

synthesis in both the T cell and pre-B cell system may share a common regulatory mechanism.

A study carried out by Rahmsdorf and colleagues (1982) did not concern Tdt, but was interesting and perhaps even relevant. They identified a protein of 35,000 D which they called XM-1. This basic, B cell specific protein is unusual in that it is expressed at a high rate in resting splenocytes, but upon polyclonal stimulation of B cells, or in proliferating B cell derived lines, XM-1 is turned off. It can be "reinduced" at this point by various agents which inhibit DNA synthesis, such as hyrdroxyurea, ultraviolet or gamma irradiation, or mitomycin C. So here is a protein which is lymphoid specific, and synthesized mainly in the absence of active DNA replication.

The induction of Tdt by the addition of exogenous ATP (Table 3-1) is an effect which has not been noted previously. There are some interesting effects which have been attributed to ATP which may be relevant to Tdt induction, but these have not been pursued in detail. Rozengurt et al. (1977) have described profound membrane pertubations induced by exogenous ATP addition to cultured cells. Among these are an increased efflux of intracellular nucleotides, especially uridine and adenosine in murine 3T6 cells. Other effects noted by

many laboratories included effects upon cell volume, histamine secretion, neurotransmission, and ion flux effects (enhanced Ca^{+2} influx, for example). The effects were specific for ATP, and were not elicited by other adenosine compounds (ADP, 5'-AMP), nor by other purine or pyrimidine nucleotides (Dubyak and De Young, 1985).

The most noteworthy effect of ATP with respect to Tdt probably concerns the enhanced Ca^{+2} influx. The effect has been seen in many cell types, including Ehrlich cells, Friend erythyroleukemia cells, and normal thymocytes (Lin et al., 1985). Both calcium and cyclic AMP are regarded as second messengers (reviewed in Berridge, 1975; Kretsinger, 1979). The activation by each (or at least signal transduction) has been determined to be mediated through the hydrolysis of phosphorylated intermediates--GTP in the case of cAMP, and phosphatidylinositol for calcium. Both compounds have been shown to function in the hormonal control of glycogenolysis (both messengers activate phosphorylase kinase). Calcium has also been shown to mimic the action of cAMP in the thyroid. It would be interesting to investigate the effects of various calcium ionophores upon Tdt expression in the FLE cells, to better understand the nature of this ATP mediated induction. established.

The identification of several agents which failed to elicit Tdt induction in FLE cells is regarded by the author to be noteworthy. Of course, the possibility always exists in experimentation of this sort that some co-factor may be needed, or that one of these agents may only potentiate the effects of a separate positive regulator of the gene, yet a broad concentration range of the various agents was tested, based upon previously published reports that documented some type of biological response in similar cell types. While current data do not support these agents as mediators of Tdt gene activation, the negative results obtained may prove equally illuminating, particularly in light of the rationale behind their testing.

Prostaglandins were investigated based upon several interesting findings. The fact that prostaglandins often act to increase intracellular cAMP levels has been well documented (Butcher and Baird, 1968; Bourne, 1974; Winkelstein and Kelley, 1980). Scheid and coworkers (1978) reported that T lymphocyte precursors exhibit transient, yet marked cAMP fluxes and T cell maturation shortly after addition of PGE_1. This, in keeping with the notion of a differentiative role of cAMP, may indicate that prostaglandins are capable of inducing differentiation in certain primitive lymphocyte series.

Another report which prompted testing of this agent was one by Goldschneider (1982) which stated that the highest source of prostaglandins (especially PGE_1) in both the human and the mouse is found in the thymus. Tdt, being an enzyme which is predominantly (if not exclusively) found in the thymus, may require just such a signal. Perhaps the PGE_1 acts as a signal which can specifically effect a differentiation pathway (via cAMP) which includes Tdt as a product. While our inducible cell line was of the pre-B lineage, it was thought that perhaps such a signal could be effective in either lymphocyte lineage, due to the early differentiative stage of the FLE cells. The agent was tested in three separate experiments, over a six-log concentration range, and uniformly failed to elicit Tdt mRNA production as evidenced by Northern blot analysis. The fact that the agent was not effective in our trials, however, does not preclude a role in normal thymocyte differentiation, or for that matter in normal thymic Tdt production. The signals for Tdt induction may vary in different micro-environments, or may be affected by cell-lineage specific sensitivites. Most likely is the possibility that the FLE pre-B cell line tested does not possess the prosta-glndin receptors present on more mature B cells and thymocytes.

The testing of cellular stress agents (heat shock conditions, ethidium bromide, and gamma irradiation) was undertaken in the hopes of defining a more definitive role for Tdt. At the time of testing, the role of Tdt in N-region generation was not demonstrated, so the enzyme remained an activity in search of a function. The basic idea was that Tdt may represent a last-resort enzyme; that is, when the cell is presented with adverse physiological conditions, it is capable of activating a final function to ensure its replication and division (the teleological goal of cellular existence?). Perhaps, if the DNA template is irrepairably damaged, or the normal replicative polymerases are inhibited, then Tdt may be able to function, with the thought that even error-prone replication is superior to no replication at all.

The rationale is not quite as superficial as the above arguments, however. Previous reports had indicated that several DNA damaging agents of the type which cause strand breakage or fragmentation increase intracellular cAMP levels (Fertel et al., 1981; Albrightson et al., 1985). So the hypothesis tested was that agents which can effect DNA breakage events may increase cellular cAMP levels, and perhaps activate the Tdt gene. None of the cellular stress agents elicited Tdt expression. Gamma

irradiation and ethidium bromide were tested in incremental doses, until toxic levels were reached.

These findings suggest that there is probably not a a "stress-induced" mechanism of Tdt expression. Several other compelling arguments support these data. Firstly, the pattern of gene expression does not resemble many known stress induced gene families. The cAMP-dependent, hormonally controlled genes of metabolism are by neccessity readily activated. They are able to be turned on and off in a matter of minutes, or even seconds. Thus, the cascade of activating steps is short, and most likely represents the conservation of a primitively acquired system of gene activation. Tdt exhibits a slower onset of activation in response to cAMP changes, representing a more finely tuned, and probably more evolved (less conserved) system of regulation. While cAMP mediation is evident in taxonomic levels as primitive as procaryotes and Dictyostelium, it is of the older, rapid onset type. The cAMP slow cascade as I will refer to it, has only been described in higher eucaryotes. Tdt itself is only seen in higher taxonomic species, suggesting a more evolutionarily advanced gene (neccessitating a more advanced level of regulation). This fact is interesting in view of the supposed role of Tdt in the generation of immunological diversity--the appearance of immuno-

globulins precedes the appearance of Tdt, phylogenetically by many levels (is the appearance of immunologic variability itself a late event?).

Another distinctive feature of cellular stress related genes is that of rapid induction, even if their action is not mediated through cAMP modulation. Heat shock genes are representative of such a gene family. The induction of these specialized proteins occur in a short time following high temperature stress. Experimentation in FLE cells revealed no activation of Tdt transcription following incubation of the cells at $42^{o}C$ for 1, 2, or even 10 minutes. In view of the potential for uncontrolled mutagenesis and cellular chaos, the control over the Tdt gene might understandably be tight.

The Role of cAMP in Tdt Induction

The present study has focused upon the enhancement of Tdt expression that various agents which elevate cAMP produce. This work reaffirms the findings of many investigators by showing that various agents which elevate cAMP levels can affect gene transcription (reviewed in Chapter I). We have extended these previous findings by demonstrating that agents which act via different biochemical mechanisms to elevate the intra-

cellular cAMP concentration have a differential ability to enhance Tdt gene expression.

Agents such as PGE_1, epinephrine, and cholera toxin have previously been shown to increase cAMP levels in various cell types (Scheid et al., 1978). Their mode of action relies upon the interaction with a specific cell surface receptor, and the subsequent activation of adenyl cyclase. Failure to elicit enhanced cAMP production (or effects attributable to such an increase) may simply reflect a lack of surface receptors for such agents. Caffeine (and other substituted xanthines) is a potent inhibitor of phosphodiesterase, a cAMP metabolizing enzyme. Dibutyryl cAMP, a derivitive of cAMP represents a third method of studying the effects of elevated intracellular cAMP levels. In many systems, the use of derivitized cAMP has been shown to be better than cAMP itself, owing to its effectiveness in traversing the cell membrane (Waymire et al., 1972), resistance to phosphodiesterase degradation (Blecher, 1971), inhibition of phosphodiesterase leading to enhanced levels of endogenous cAMP (Heersche et al., 1971), or even their direct activation of protein kinase (Blecher, 1971; Wagner et al., 1975).

We have shown that various agents which elevate intracellular cAMP concentrations via different

mechanisms can effect an accumulation of Tdt mRNA. These data suggest that agents which alter cAMP levels might regulate immune function. The intracellular cAMP assay data do reveal some clue as to a mechanism of activation of the Tdt gene. The intracellular cascades associated with cAMP-mediated gene expression are just beginning to be understood (see Chapter I). Many of the earliest examined cAMP-associated events were shown to occur within a very short time after addition of some exogenous effector, but this rapid activation may not be the exclusive mechanism of cAMP mediated events. Tata (1986) documented another type cAMP "cascade" which has a somewhat slower onset, and longer duration. The classes of genes which are activated tend to relate to the immediacy of the cellular need for that gene product. For example, the need to increase gluconeogenesis in the absence of available glucose is an immediate one in order to ensure adequate energy for cellular metabolism. The action of glucagon, the polypeptide hormone responsible for such energy production, is mediated through the action of cAMP on target tissues. Such responses have been referred to as biochemical "reflexes," due to the rapidity of onset. Terminal transferase, as an example of a normal, non-metabolic enzyme, may not demand immediate activation. Rather, a gradual quiescence and

cAMP accumulation accompanying pre-B differentiation could be responsible for Tdt gene transcription. The exact mechanism by which this effect is achieved has not been elucidated, yet some preliminary findings with the Tdt gene, as well as numerous reports of cAMP regulation of other gene products, point to a role of protein phosphorylation in gene expression.

Cell cycle kinetics during dbcAMP/caffeine induction were monitored using flow cytometry (Figure 3-27). The changes observed during a 24 hour induction are characteristic of cAMP effects on cell growth; namely, a gradual slowing of cell progression from G_0/G_1 to S phase. Control cultures show a modest increase in the percentage of cells entering S phase with time, as expected. Yet these cAMP-dependent changes in cell cycle kinetics are so modest, that they tend to argue against a phase-specific expression of Tdt. Indeed, previous studies have shown that Tdt expression is unaffected by cell cycle stage in continuously growing leukemic cells (Harrison et al., 1976). The cytotoxic effect of hydroxyurea is apparent from the figure (3-27), with a preferential S phase cytolytic event, yet parabactin, which induced the enzyme just as well, failed to show such an effect. Although it is possible that the expression of Tdt is an event limited to a small propor-

tion of the cell population which is gradually expanded, it should be remembered that the cells have been cloned and sub-cloned several times, and represent a homogeneous cell population (at least as homogeneous as transformed cells may be). In addition, when cells are maintained in inducing agents for extended periods, the level of Tdt expressed remains high and constant, even during periods of extreme crowding, when cell cycle effects may manifest themselves. At this point, the only valid conclusion which can be reached is that various inhibitors of DNA synthesis can induce the synthesis of terminal deoxynucleotidyl transferase. Preliminary results suggest that increases in intracellular cAMP concentrations accompany this DNA synthesis inhibition, and may play a role in Tdt gene transcription in FLE cells. This increased level of Tdt-specific mRNA seems to be accompanied by a concomitant increase in the degree of phosphorylation of histone H1.

A speculative hypothesis currently favored by the author attempts to explain the diverse occurrence of N-regions, those short stretches of DNA apparently created de novo by the action of Tdt. They have been described in the immunoglobulin locus (where extensive sequencing has ruled out a germline origin of the nucleotide stretch), T cell locus, Burkitt lymphoma (EBV-trans-

formed) cell lines, and most recently in yeast. At first glance these systems seem quite disparate, yet there is an apparent commonality. In each of the aforementioned systems, DNA rearrangements in the form of genetic translocations have taken place. Specifically, all of these examples involve a double-stranded DNA breakage and rejoining event. The break-rejoin process occurs either as a stage in the course of normal differentiative recombination events (B and T cell receptor loci, yeast mating type cassettes), or as chromosomal translocations in certain tumor cell lines. These strand breakage events may act to increase intracellular cAMP levels, as previously documented with exogenously supplied DNA damaging agents (Fertel et al., 1981; Albrightson et al., 1985). Transcription of Tdt may then begin in response to such a cAMP increase. The problems with testing such a theory are numerous. Tdt may be turned on transiently for a very brief time period, and perhaps only in certain susceptible cell types. Tight control over Tdt biosynthesis and activity are imperative in order to avoid uncontrolled mutagenesis. The notion that the expression of Tdt may be linked to DNA ligase activity, and thus work to repair and restore damaged tracts of DNA, had been examined by David et al. (1985). They reported an inverse relationship between Tdt activity and ligase

activity in human ALL cells. Although the goal of that study was to develop clinical correlates to leukemic cell biochemical markers (such as Tdt), they do mention the possibility of a sequential expression of Tdt and then DNA ligase.

Another problem with testing the theory of DNA breakage induction of Tdt activation concerns the availability of an appropriate intracellular assay for the enzyme's activity. Yancopoulos et al. (1986) utilized plasmids consisting of germ-line (unrearranged) T cell receptor gene segments, and transfected them into cell lines representing various early stages of B lymphocyte development--including both Tdt positive and negative cell lines (see introduction). Examination of the inserted sequences (after several rounds of replication and division) revealed correct joining of the segments, plus the additional N-region stretches in those cell lines which were positive for Tdt. Landau et al. (1987) did a similar experiment using a retrovirally inserted Tdt cDNA in otherwise Tdt^- cells, and observed N-region formation with a retrovirus-based artificial immunoglobulin gene rearrangement substrate, in lines expressing the Tdt cDNA (see Chapter I). Thus, a strong positive correlation between the presence of N regions and Tdt has been noted and for the most part accepted in

the scientific community. The experiments have only been done in constitutive producers of Tdt, and thus may not be a totally accurate or for that matter relevent depiction of the in vivo scenerio. In addition, no other rearranging plasmids have been tested. It would be interesting to examine the range of Tdt activity. That is, is the activity of the enzyme limited to lymphoid (or immunoglobulin genes) or are we seeing a non-specific effect limited solely by the appropriate DNA configuration. If this latter situation proved to be the case, then the arguments for a tight regulatory mechanism governing the Tdt gene expression become more compelling. It is conceivable that the Tdt gene is a part of a developmental program involved in lymphoid gene rearrangement. Perhaps the gene is controlled by a promoter, or regulatory network which also controls (or responds to) expression of specific recombinases involved in B and T cell receptor rearrangement. A careful examination of the context, or at least the upstream control regions of the gene in lymphoid and non-lymphoid cells may prove illuminating in this regard.

Future Concerns

The future of Tdt research is limited only by the imagination of the workers in the field. The fact that several inducible cell lines have been isolated and detected will enable researchers to perform meaningful *in vitro* experiments, such as analyses of inserted templates following Tdt induction. Knowledge of the molecular mechanisms which govern Tdt induction (such as DNA inhibition) will lead to the testing of relevant compounds *in vitro*. A careful survey of such agents will lead to the search for *in vivo* correlates, or naturally-produced agents which produce the same result in an organ or microenvironment.

Specific proposals for the continuation of this research project should be designed to better elucidate the action and mechanisms of DNA synthesis inhibitors upon the expression of Tdt. A discrimination of the type of inhibitors effecting Tdt expression may prove useful. For example, compounds which inhibit DNA synthesis at different metabolic sites (other than ribonucleotide reductase) should be tested. In addition, a confirmation of the cAMP induction seen with hydroxyurea should be performed, and extended to other agents which induce Tdt.

The subtractive hybridization scheme proposed as a method for cloning the Tdt gene may have proven useful in establishing a library of genes which are co-expressed (or co-induced) with Tdt. This project is still a viable one, and could yield important information, especially with respect to DNA binding proteins and recombinases which may be involved in both B and T cell differentiation. The notion of cAMP induced gene families is not unfounded. They have been noted in systems as diverse as Dictyostelium (Williams et al., 1980), mammary carcinoma cell lines (Huang et al., 1984), and teratocarcinoma cells (Strickland et al., 1980), and have not been examined in the primitive lymphoid system.

Terminal deoxynucleotidyl transferase is unique among enzymes, in that it represents an activity in search of a function. Inevitably, a definitive role for Tdt will be assigned and accepted. As is the case in all of science, the solving of a mystery only paves the way for more questions.

"The most beautiful thing we can experience is the mysterious. It is the source of all true art and science"

--Albert Einstein

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BIOGRAPHICAL SKETCH

Randy Jonathan Horwitz was born on November 30, 1958, in Chicago, Illinois, the same city in which he was conceived, about nine months earlier. His formative years were spent in Skokie, Illinois, where he attended Niles West High School.

Randy attended the University of Illinois at Champaign-Urbana, until 1980, when the administration offered him a B.S. in Biochemistry if he promised never to return. He hitched south to the University of Florida in Gainesville, and enrolled in graduate school in the Department of Immunology and Medical Microbiology. He began working in the laboratory of Edward Siden in 1981, and remained there until 1986, when Randy enrolled in medical school back at the University of Illinois, still located in Champaign-Urbana.

He is currently a second year medical student, at the Rockford campus, where he is torn between academic failure and completion of his dissertation. With any luck, and perhaps Divine intervention, he will receive his Ph.D. in 1988, his M.D. in 1990, and his Loan Repayment Schedule in 1991.

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

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I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

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I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

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I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

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